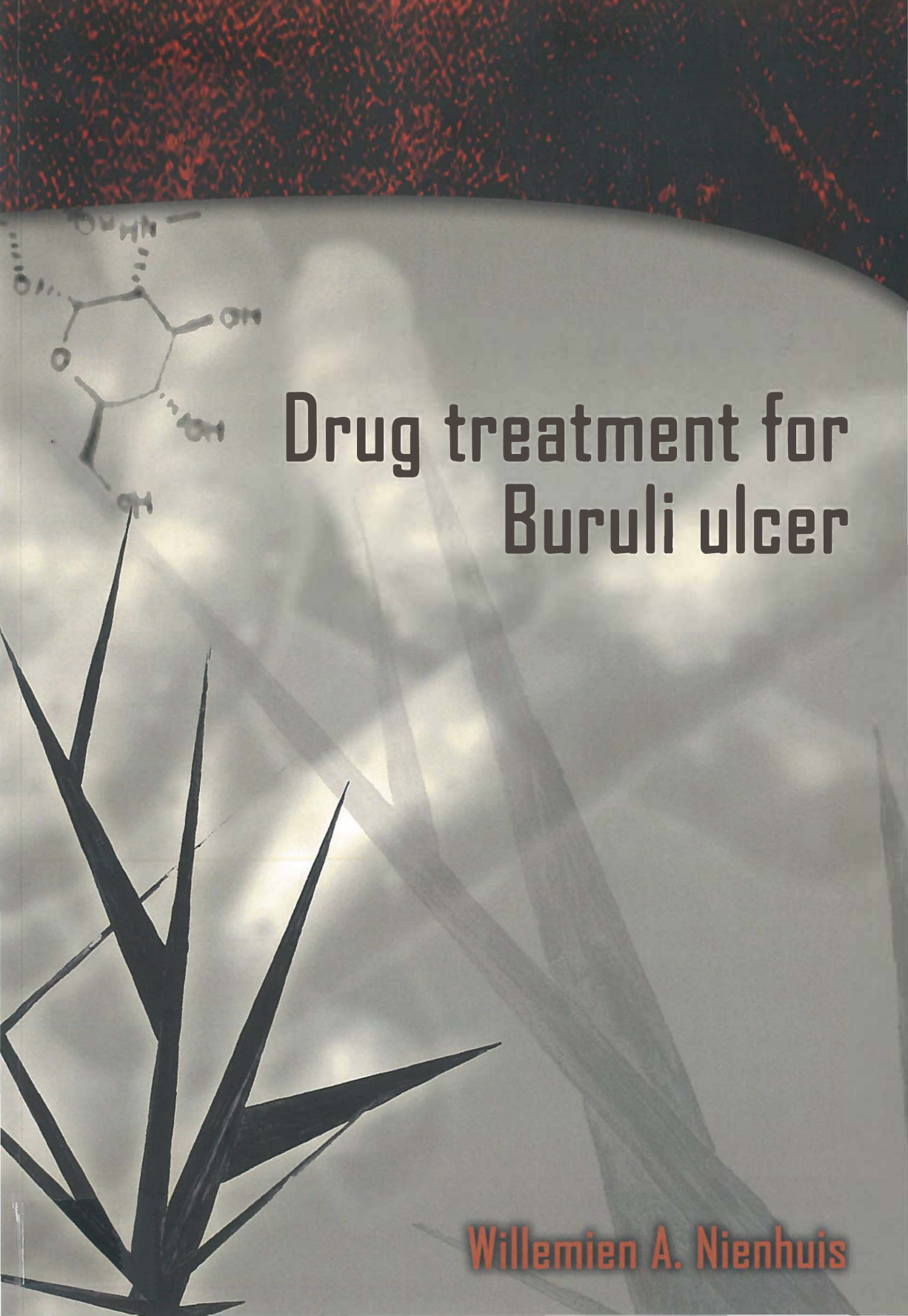


Drug treatment for Buruli ulcer

Willemien A. Nienhuis

Drug treatment for Buruli ulcer

STELLINGEN

BEHOREND BIJ HET PROEFSCHRIFT

DRUG TREATMENT FOR BURULI ULCER

1. The WHO guidelines on treatment of Buruli ulcer should be changed in that injected aminoglycoside can now be reduced from eight to four weeks *(this thesis).*

2. Paradoxical responses after start of antimicrobial treatment for Buruli ulcer explain why the effectiveness of antibiotics has not been recognized earlier *(this thesis).*

3. Until a sensitive and specific test to monitor response to Buruli ulcer treatment has been developed, clinical judgment of the lesion is more important than consecutive surface area measurement to differentiate a paradoxical response from treatment failure *(this thesis).*

4. The preponderance of Buruli lesions on one side of the body should be explored for possible routes of transmission *(this thesis).*

5. In non-ulcerative Buruli ulcer lesions, ulceration is not prevented by antimicrobial treatment as it occurs in the majority of cases *(this thesis).*

6. Financiering van klinisch wetenschappelijk onderzoek naar 'Neglected Tropical Diseases' zou hoger op de ranglijst dienen te staan bij subsidie verstrekkers.

7. De waarschijnlijk uit Afrika afkomstige, maar in Nederland ingeburgerde zegswijze 'het vergt een heel dorp om een kind groot te brengen', wordt aldaar nog gewaardeerd en nageleefd, dit in tegenstelling tot in Nederland.

8. Door vol te houden bereikte de slak de ark *(Charles Haddon Spurgeon, By perseverance the snail reached the ark).*

9. De kunst is niet alleen je eigen hersenen te gebruiken, maar ook alle die je kunt lenen *(Woodrow Wilson, I not only use all the brains that I have but all that I can borrow).*

10. De p-waarden zeggen of de getallen liegen, het waarheidsgehalte zegt of het artikel liegt *(Yvo Smulders).*

11. Het gezegde 'niet over een nacht ijs gaan' gaat niet op voor Hollanders die direct een optie nemen op een hotelkamer in Leeuwarden, zodra het een nacht gevroren heeft *(Cornelis Halma).*

12. Het leven is als een multiple choice vraag: hoe meer keuzemogelijkheden, hoe moeilijker.

13. Wanneer de deadline is gehaald komt de promovendus weer tot leven.

14. Sa is 't en net oars, want as it oars wie, wie 't net sa *(Friese uitdrukking).*

Willemien A. Nienhuis, Leeuwarden, april 2012

Studies presented in this thesis were financially supported by: European Union, Jan Kornelis de Cock Stichting, GSMS (Groningen Graduate School of Medical Sciences), Netherlands Organisation for Scientific Research and Stichting BuG (Buruli ulcer Groningen Foundation). Publication of this thesis was financially supported by: RuG (Rijksuniversiteit Groningen) and GSMS (Groningen Graduate School for Drug Exploration). All contributions are gratefully acknowledged.

Cover and chapter paintings by Marie-Anne Franqueville: her painting *"Cristal de lames"* was used to represent streptomycin crystals.

ISBN: 978-90-6464-543-3

Cover design: A.W. Kampen en W.A. Nienhuis
Paintings: Marie-Anne Franqueville
Layout: Ferdinand van Nispen, CitroenVlinder DTP & Vormgeving, Bilthoven
Printed by: GVO drukkers & vormgevers B.V. | Ponsen & Looijen, Ede, The Netherlands

RIJKSUNIVERSITEIT GRONINGEN

Drug treatment for Buruli ulcer

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen

aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 4 april 2012
om 11.00 uur

door

Wilhelmina Anjelina Nienhuis

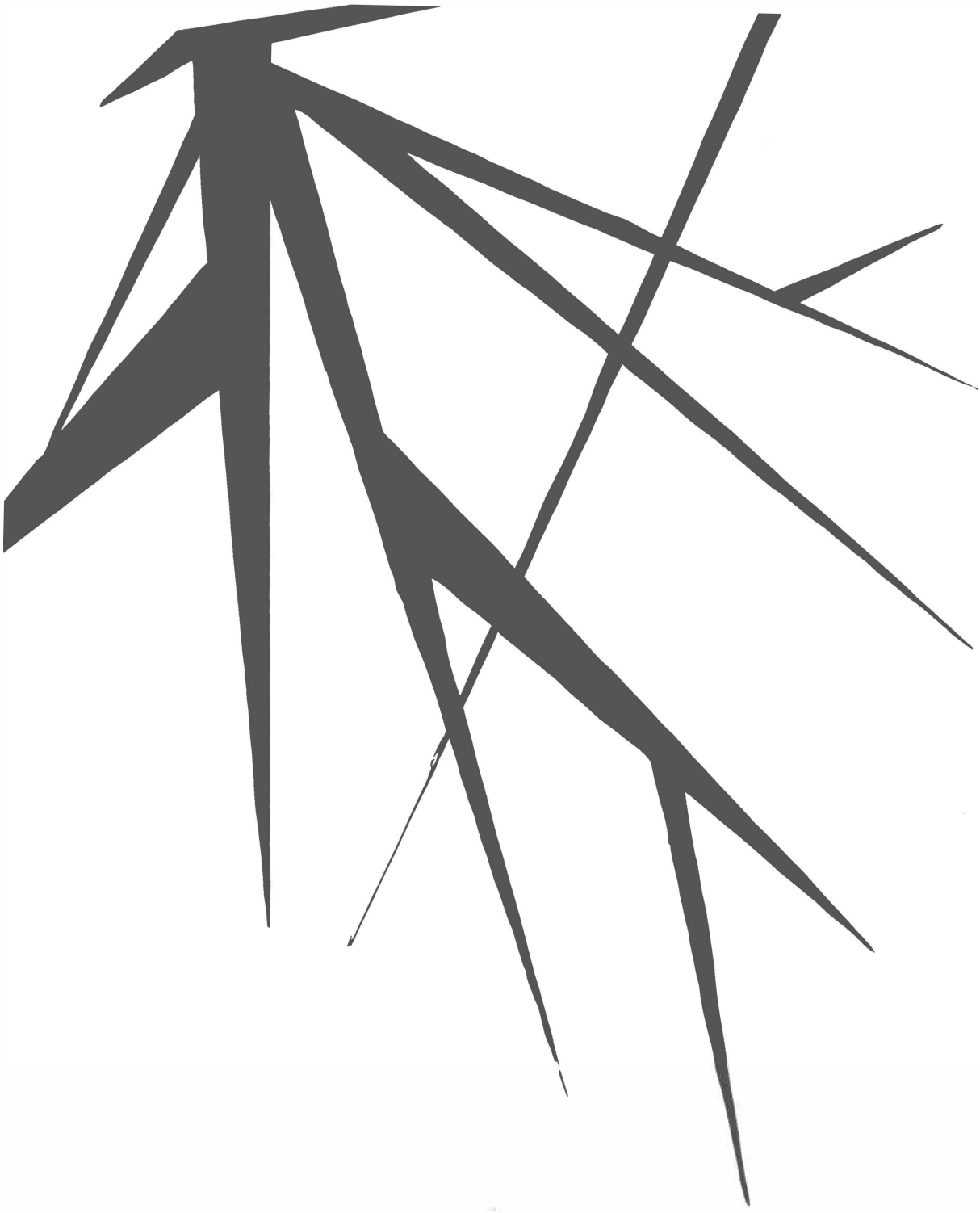
geboren op 6 april 1978
te Buitenpost

Promotor:	Prof. dr. T.S. van der Werf
Copromotor:	Dr. Y. Stienstra
Beoordelingscommissie:	Prof. dr. W.R. Faber Prof. dr. B. Fleischer Prof. dr. B.C. de Jong

Paranimfen: Sandra Boekestein-Loves
Esther Stroetenga-Vis

For the BU teams and patients,
Agogo & Nkawie hospital

Contents



CHAPTER 1

General introduction

Drug treatment for Buruli ulcer

Buruli County was once the name of a district in central Uganda near the Nile river. This district is now called Nakasongola, after the name of its major town (figure 1). Half a century ago, a report was published in the Lancet about skin ulcers occurring in that region [1]. The authors called the causing agent of the skin ulcers *Mycobacterium buruli* [2]. Some years hereafter, refugees from Rwanda who moved to Uganda into a settlement located near a swampy area in the Nile river basin also developed extensive and crippling ulcers [3]. Earlier, in the 1950s, several reports came from Zaire –now DR Congo–, describing necrotizing skin lesions with large loads of acid-fast bacilli [4,5]. All of these reports addressed the disease now commonly called Buruli ulcer disease or *Mycobacterium ulcerans* infection. The first description in West Africa was from Nigeria in 1967 [6]. In 1971, two cases were reported from Ghana [7]. Since then the disease has emerged in western parts of Sub-Saharan Africa. Reports have however come from over 30 countries around the world. The first report with a clear description of the clinical presentation and histopathology identifying the causative pathogen, dates back to 1948. In this report from Australia it was shown that a slowly growing mycobacterium could be cultured around 30°C, and that the subsequent inoculation of these bacteria in experimental animals caused similar pathology [8]. Cases of Buruli ulcer have also been reported from scattered foci in Asia and South America [9-11].

The micro-organism is generally believed to be an environmental mycobacterium residing in swampy, riverine areas in humid climates, but the reservoir is not fully understood [9]. Likewise, the exact mode of transmission is still unknown [12]; person-to-person transmission is however extremely rare [13]. Circumstantial evidence suggests that trauma introduces the etiologic agent into the skin and the subcutaneous tissue [14-17].

Mycobacterium ulcerans infection usually starts as a nodule, papule, plaque, or edema (figure 2-4). When the lesion breaks open, a typical painless ulcer with undermined edges appears (figure 5), which can progress to a large necrotic wound. Lesions predominate on the limbs, and children of school-going age, residing in remote areas, are most often affected. The disease can be self-limiting [9,18,19], but leaves patients often with large scars and deformities. Stigmatization is still common, and is one of the reasons for delay in reporting [20,21]. Even large lesions rarely cause pain or systemic symptoms, which is another source of delay in seeking treatment early [22,23].

Since its first description, different treatments have been investigated, but surgery has dominated. Even Albert Cook, who described lesions we now recognize as

typical for Buruli ulcer as early as 1897 [24,25], tried surgical removal of affected tissues. In Australia MacCallum *et al.* reported surgical treatment with subsequent healing [8]. In 1964 Lunn *et al.* concluded from several observational reports that '*in all these, chemotherapy alone was ineffective as a sole means of treatment, though varying degrees of activity against the causative mycobacterium have been demonstrated in vitro*' [26]. In 1970, the Uganda Buruli Group reported that '*we do use antimycobacterial drugs for hospital patients, but it seems that the most important factor in healing is surgical technique*' [27]. Surgery cannot completely remove all bacilli. Recurrence is common, with reported rates varying between 6% [28] and 47% [29]. Although larger excisions might be more effective, the inherent tissue damage increases duration of treatment

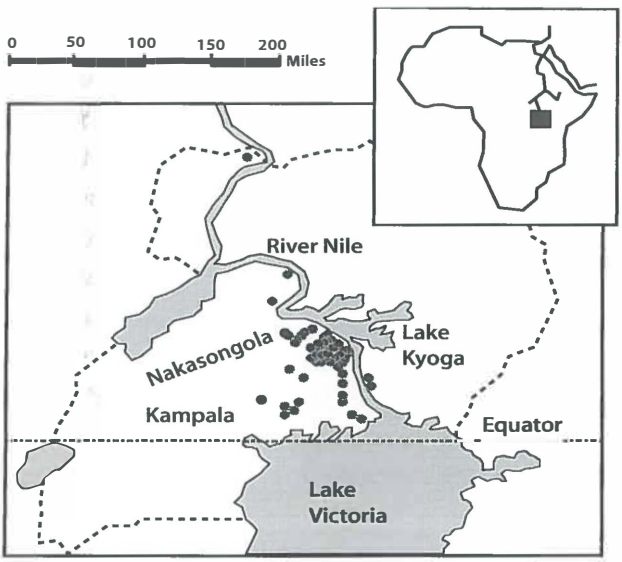


Figure 1: Map of Uganda showing the high incidence of mycobacterial ulcers in de the Buruli area. Patients' homes are shown with a dot. With permission from *Clancey et al., Mycobacterial skin ulcers in Uganda, the Lancet 1961*

Figure 2: Nodule above the elbow

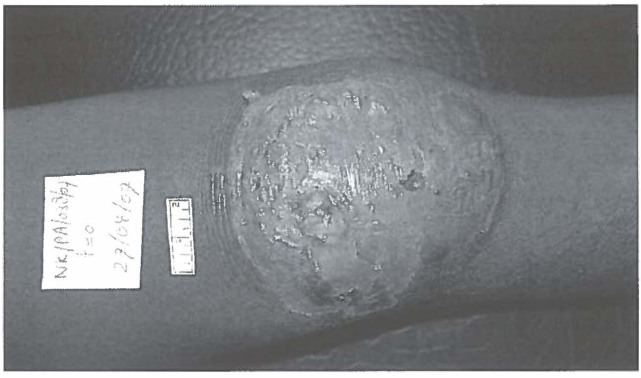


Figure 3: Plaque on the knee

Figure 4: Edema of the right arm

Figure 5: Ulcer on the left shoulder

and chances of residual functional limitations. Many antimycobacterial agents show activity against *Mycobacterium ulcerans* in vitro and in animal models [30-40]. As early as in the 1950s and 1960s, success was reported with clofazimine and with streptomycin for the treatment of early experimental infection in mice [26,41,42]. In 1972 the in vitro sensitivity of *Mycobacterium ulcerans* to rifampicin was found to be similar to that of *Mycobacterium tuberculosis* [43]. Heat therapy in combination with rifampicin yielded good results in the mouse footpad infected with *Mycobacterium ulcerans* [44]. Two randomized placebo controlled trials have been carried out using clofazimine and dapsone plus rifampicin. Neither of these trials showed a difference in rate of healing between groups [45,46]. Chemotherapy was generally disappointing. Extensive surgical debridement became the standard, with or without subsequent skin grafting. In 2003 a study in Ghana was conducted in patients with pre-ulcerative (nodular) lesions. The number of study participants was relatively small, and only 21 had their *Mycobacterium ulcerans* disease confirmed by PCR testing. The study participants received variable periods of streptomycin plus rifampicin treatment. All lesions were surgically removed, and histopathology, PCR and culture was performed of all resected tissues. In some of the lesions *Mycobacterium ulcerans* could be cultured, but in none of the tissues of affected individuals receiving drug treatment of 4 weeks or more, showing that *Mycobacterium ulcerans* can be killed in human tissues by drug treatment alone [47]. Antibiotics were used erratically in regions

where surgery was not easily accessible, with the imminent danger of developing resistance resulting from mono-therapy or sub-therapeutic dosage drug treatment [48]. Therefore WHO issued preliminary guidelines for the drug treatment of Buruli ulcer disease in 2004, with 8 weeks of standard antibiotic treatment, with or without surgery [49]. In an observational study reported in 2007, promising clinical efficacy of the combination streptomycin and rifampicin was reported from Benin, where close to 50% (102 out of 224) of the included Buruli ulcer patients were treated successfully with antibiotics alone. Of those, two recurrences were reported after one year. In the other patients it was judged necessary to add surgical treatment; in 113 successfully treated patients one recurrence was reported after one year [50]. Buruli ulcer disease is one of 19 neglected tropical diseases addressed by WHO in its *Global plan to combat neglected tropical diseases 2008–2015* [51]. Here the organization describes *Mycobacterium ulcerans* infection as a disease for which there are no cost-effective control methods. This thesis addresses drug treatment for Buruli ulcer.

Should antibiotics be given for Buruli ulcer?

Does treatment using streptomycin and rifampicin given for 8 weeks, without the use of large debridement surgery, result in healing of *Mycobacterium ulcerans* infection without recurrence of disease? Is an oral regimen using clarithromycin instead of streptomycin during the second 4 weeks inferior compared to 8 weeks of injection therapy?

In Chapter 2 we test the hypothesis that early (duration less than 6 months), limited (lesion cross-sectional diameter smaller than 10 cm) *Mycobacterium ulcerans* infection can be cured with 8 weeks of antimycobacterial therapy, without adding debridement surgery. Studies in animal models showed that aminoglycosides – e.g. streptomycin and amikacin – and rifampicin have strong bactericidal activity [33-35]. The most harmful side effects of aminoglycosides are ototoxicity and renal toxicity [52-54], and administration is contra-indicated during pregnancy because of potential toxic effects on the fetus. In highly endemic areas such as Sub-Saharan Africa, hygiene is a concern as aminoglycosides can only be administered through injections [55-58].

We therefore tested a second hypothesis – i.e., whether switching injected streptomycin to oral clarithromycin after 4 weeks would be equally effective, or at least, non-inferior to the streptomycin-based therapy. Earlier, clarithromycin showed bacteriostatic and weak bactericidal activity in vivo when the study was designed [34,35].

Clarithromycin for Buruli ulcer

Does rifampicin co-medication result in lower serum concentrations of clarithromycin when treating *Mycobacterium ulcerans* infection? What is the additional effect of the metabolite 14-hydroxyclarithromycin (14-OH clarithromycin) in the treatment of *Mycobacterium ulcerans* infection?

Co-medication can result in significant drug-drug interaction. This can be so profound that a decrease to sub-therapeutic serum concentrations might result. Rifampicin is known to induce cytochrome P450 (CYP) enzymes, which are responsible for the hepatic metabolism of many active drugs [59-61]. As CYP3A4, one of those enzymes, is involved in the elimination of clarithromycin [61,62], a faster metabolism into its major – and, possibly also active – metabolite 14-OH clarithromycin will result [63]. Clarithromycin, on the other hand, is known to inhibit CYP450 enzyme systems [60,64,65]. Chapter 3 explores the drug-drug interaction between rifampicin and clarithromycin in a subset of patients that participated in the randomized trial described in chapter 2. The extent of the drug-drug interaction of these two drugs had not been studied earlier in this population and with the present prescription used (clarithromycin 7.5 mg/kg of body weight, once daily; rifampicin 10 mg/kg, once daily). Furthermore, in chapter 3, minimal inhibitory concentrations (MICs) of clarithromycin and 14-OH clarithromycin are determined for a Ghanaian and a Malaysian *Mycobacterium ulcerans* strain. For 14-OH clarithromycin the activity against *Mycobacterium ulcerans* has never been described, and it is unknown to what extent the antimycobacterial effects of the metabolite add to activity of clarithromycin itself. We hypothesized that despite the described drug interactions, effective inhibitory concentrations of both combined drugs would be achieved for at least some period of time [66], and that therefore no inadvertent mono-therapy would jeopardize the effectiveness of treatment with possible selection pressure allowing naturally occurring drug-resistant mutant organisms to repopulate Buruli ulcer lesions [48,67].

Response patterns to drug treatment of Buruli ulcer

Why have antibiotics long been thought to be ineffective for the treatment of *Mycobacterium ulcerans* infection? Is there evidence for a paradoxical response accompanying antimicrobial treatment for Buruli ulcer disease?

When carrying out the randomized controlled trial described in chapter 2, we were struck by the fact that, overall, healing was slow. Moreover, in quite some patients it looked like healing set in after an initial worsening of disease had been noticed. This led to the hypothesis of a paradoxical reaction accompanying effective mycobacterial killing. In the past, paradoxical reactions might have been misunderstood as treatment

failure [68,69]. This could, in part, explain why the effectiveness of antimicrobial therapy for Buruli ulcer disease has not been recognized earlier. Paradoxical reactions are well known in other mycobacterial diseases, particularly tuberculosis [70-72]. After starting anti-tuberculosis treatment, immediate effect can be observed by a decrease in symptoms. Despite a possible paradoxical reaction, treatment is likely to be continued, as the phenomenon is recognized as such, and not misinterpreted as treatment failure. A paradoxical reaction in (pulmonary) tuberculosis might be less obvious than in a skin disease like Buruli ulcer, where treatment responses are observed continuously in case of non-ulcerative lesions, or whenever dressing changes allow observation of the lesion, as is the case in ulcerative stages of disease. Besides, initial improvement may be more subtle in *Mycobacterium ulcerans* infection, as the disease is not associated with systemic symptoms, unless secondary infection is present. Early surgical intervention might have biased results of clinical trials and observations on antibiotic treatment for Buruli ulcer. Chapter 4 describes disease response patterns after start of antibiotic therapy for Buruli ulcer disease in patients who healed on therapy, intending to find support for the hypothesis of paradoxical reactions accompanying antimycobacterial killing.

Streptomycin for Buruli ulcer – ototoxicity screening in the field

Can screening for aminoglycoside-induced ototoxicity be reliably performed in the field?

Measurement of hearing levels can be used as a screening method for developing hearing impairment as a result of streptomycin toxicity [52,73]. Chapter 5 addresses the feasibility and reliability of measuring hearing levels using portable audiometry in a field hospital setting. We hypothesized that this screening method can be reliably used in hands of inexperienced, briefly trained health personnel, preventing the need for sending patients to specialized centers for audiometry, which are often far away, and not easily accessible.

Genetics and vitamin D in Buruli ulcer

Does serum vitamin D concentration influence susceptibility to develop Buruli ulcer disease, or the response to antibiotic treatment? To what extent do genetic polymorphisms in the vitamin D receptor, or in other candidate genes, play a role in susceptibility to develop Buruli ulcer disease after *Mycobacterium ulcerans* infection, and do genetic polymorphisms play a role in response to treatment?

Vitamin D has been considered as a treatment option in tuberculosis in the pre-antibiotic era, along with other supportive measures [74]. Later it was shown that

metabolites of this hormone-like molecule are essential in the antimicrobial immune reaction cascade, triggered upon contact with certain pathogens that enter the human body, like mycobacteria [75,76]. Vitamin D effects depend on the presence of adequate stores, best measured by blood concentrations of 25-hydoxyvitamin D [77,78], and on the genetically determined susceptibility of the vitamin D receptor [79]. Most tissues and cells in the body have vitamin D receptors, and the majority of immune cells, notably monocytes and macrophages, have the ability to convert vitamin D into its active form, 1,25-dihydroxyvitamin D [78]. In this way the supra-physiological concentrations of active vitamin D, necessary to modulate immune responses at the local level, can be reached [76]. Epidemiological evidence suggests there is a relation between vitamin D deficiency, as well as polymorphisms in the vitamin D receptor gene, and susceptibility to tuberculosis [80-84]. Certain vitamin D receptor gene polymorphisms have also been found to be associated with leprosy type (tuberculoid versus lepromatous) [85]. A study that assessed outcome of tuberculosis treatment showed an association between vitamin D receptor polymorphism and time to sputum culture conversion during anti-tuberculosis treatment [86]. In a study that assessed the potential of vitamin D suppletion in tuberculosis patients, vitamin D receptor polymorphism was associated with time to sputum culture conversion as well [87]. In summary, there is evidence to suggest that host response after mycobacterial infection is related to vitamin D status and vitamin D receptor polymorphism status. In chapter 6 we therefore studied the hypothesis that vitamin D serum concentration and vitamin D receptor polymorphism is associated with *Mycobacterium* ulcerans infection, as well, and that response to treatment depends on vitamin D status and vitamin D receptor polymorphism.

Only a certain percentage of individuals infected with *Mycobacterium ulcerans* develop overt clinical disease [88-92]. Like in tuberculosis and leprosy, a combination of environmental and inherited factors seem to determine this clinical response; apart from the vitamin D receptor gene, other genes are known to influence susceptibility to *Mycobacterium tuberculosis* and *Mycobacterium leprosy* [93-96]. For *Mycobacterium ulcerans* infection, the only published study on genetic susceptibility reported that a certain polymorphism in the SLC11A1 gene increased the chance of developing Buruli ulcer disease by 13% [97]. In chapter 6 we seized the opportunity to try confirm this finding, and apart from the SLC11A1 and the VDR gene, we studied the MBL (mannose binding lectin) gene. We tested the hypothesis that polymorphisms in these candidate genes are associated with Buruli ulcer disease in our population, and with response to treatment.

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CHAPTER 2

Antimicrobial treatment for early, limited *Mycobacterium ulcerans* infection: a randomised controlled trial

Willemien A Nienhuis
Ymkje Stienstra
William A Thompson
Peter C Awuah
K Mohammed Abass
Wilson Tuah
Nana Yaa Awua-Boateng
Edwin O Ampadu
Vera Siegmund
Jan P Schouten
Ohene Adjei
Gisela Bretzel
Tjip S van der Werf

Lancet. 2010 Feb 20;375(9715):664-72

Summary

Background Surgical debridement was the standard treatment for *Mycobacterium ulcerans* infection (Buruli ulcer disease) until WHO issued provisional guidelines in 2004 recommending treatment with antimicrobial drugs (streptomycin and rifampicin) in addition to surgery. These recommendations were based on observational studies and a small pilot study with microbiological endpoints. We investigated the efficacy of two regimens of antimicrobial treatment in early-stage *M ulcerans* infection.

Methods In this parallel, open-label, randomised trial undertaken in two sites in Ghana, patients were eligible for enrolment if they were aged 5 years or older and had early (duration <6 months), limited (cross-sectional diameter <10 cm), *M ulcerans* infection confirmed by dry-reagent-based PCR. Eligible patients were randomly assigned to receive intramuscular streptomycin (15 mg/kg once daily) and oral rifampicin (10 mg/kg once daily) for 8 weeks (8-week streptomycin group; n=76) or streptomycin and rifampicin for 4 weeks followed by rifampicin and clarithromycin (7·5 mg/kg once daily), both orally, for 4 weeks (4-week streptomycin plus 4-week clarithromycin group; n=75). Randomisation was done by computer-generated minimisation for study site and type of lesion (ulceration or no ulceration). The primary endpoint was lesion healing at 1 year after the start of treatment without lesion recurrence or extensive surgical debridement. Analysis was by intention-to-treat. This trial is registered with ClinicalTrials.gov, number NCT00321178.

Findings Four patients were lost to follow-up (8-week streptomycin, one; 4-week streptomycin plus 4-week clarithromycin, three). Since these four participants had healed lesions at their last assessment, they were included in the analysis for the primary endpoint. 73 (96%) participants in the 8-week streptomycin group and 68 (91%) in the 4-week streptomycin plus 4-week clarithromycin group had healed lesions at 1 year (odds ratio 2·49, 95% CI 0·66 to infinity; p=0·16, one-sided Fisher's exact test). No participants had lesion recurrence at 1 year. Three participants had vestibulotoxic events (8-week streptomycin, one; 4-week streptomycin plus 4-week clarithromycin, two). One participant developed an injection abscess and two participants developed an abscess close to the initial lesion, which was incised and drained (all three participants were in the 4-week streptomycin plus 4-week clarithromycin group).

Interpretation Antimycobacterial treatment for *M ulcerans* infection is effective in early, limited disease. 4 weeks of streptomycin and rifampicin followed by 4 weeks of rifampicin and clarithromycin has similar efficacy to 8 weeks of streptomycin and rifampicin; however, the number of injections of streptomycin can be reduced by switching to oral clarithromycin after 4 weeks.

Funding European Union (EU FP6 2003-INCO-Dev2-015476) and Buruli Ulcer Groningen Foundation.

Introduction

Buruli ulcer is a necrotising infection of subcutaneous tissue caused by *Mycobacterium ulcerans*.[1] The name Buruli ulcer comes from a region near the Nile River delta in Uganda, named Buruli County, where the disease was highly endemic in the 1960s.[2] Today, the disease is emerging in west African countries with thousands of cases every year, mainly in children.[3,4] A plasmid of *M ulcerans* encodes the production of mycolactone,[4,5] an immunomodulatory macrolide toxin that causes tissue necrosis.[6] *M ulcerans* is acquired near slow-flowing and stagnant water in tropical and subtropical environments. The natural reservoir and mode of transmission of the infection remain largely obscure and might differ between endemic foci around the world.[7,8] However, skin injury[9] and insect bites[10] have been proposed as modes of transmission.

M ulcerans infection usually starts as a nodule, papule, plaque, or oedema. When left alone, the lesion breaks open and a typical painless ulcer with undermined edges appears, which can progress to a large necrotic lesion. WHO has defined lesions with a cross-sectional diameter of less than 5 cm as category I, 5–15 cm as category II, and more than 15 cm, lesions on important sites (eye, breast, and genitalia), or multiple lesions as category III. *M ulcerans* infection can be self-limiting, but scar tissue and contractures in joints leave patients with functional limitations and social stigma.[11,12] The diagnosis can be made clinically but culture is the gold standard. However, this method is difficult and has low sensitivity.[1,3,4,13] Since the development of PCR targeting insertion sequence 2404 (IS2404)—a repetitive oligonucleotide unit with more than 200 copies in the genome of M ulcerans[14]—diagnostic confirmation has improved substantially.[13,15,16]

Buruli ulcer is one of 19 neglected tropical diseases addressed by WHO in its Global plan to combat neglected tropical diseases 2008–2015.[17] In this plan, the organisation describes Buruli ulcer as a disease for which there are no cost-effective control methods. Since the disease's first description in 1948,[18] different treatments have been investigated. Extensive surgical debridement, with or without subsequent skin grafting, is standard treatment. However, surgery cannot completely remove all bacilli[19] and recurrence is common, with reported rates varying between 6% and 47%.[20–22] Although larger excisions might be more effective, they can increase chances of residual functional limitations. In the first of two randomised controlled trials for *M ulcerans* infection, clofazimine did not show a significant benefit compared with placebo.[23] In individuals with small (<5 cm), non-ulcerated lesions, recurrence-free healing without surgery was reported in five of eight participants who were treated

with clofazimine compared with five of 17 who were treated with placebo. In ten patients with larger and ulcerated lesions, all except one (in the placebo group) needed surgery. A second study compared the effect of dapsone plus rifampicin with placebo. Of 41 randomised patients, 30 completed the 2-month trial. Rate of healing did not differ between groups. Uneven baseline characteristics might partly explain why patients assigned to active treatment had a larger reduction in lesion size than did patients assigned to placebo.[24]

Many antimycobacterial agents show activity against *M ulcerans* in vitro, and experiments in animals, such as the mouse footpad model, show that streptomycin in combination with rifampicin is highly bactericidal. In a pilot study sponsored by WHO, 31 patients clinically diagnosed with pre-ulcerative *M ulcerans* infection were treated with streptomycin and rifampicin for 0, 2, 4, 8, or 12 weeks.[25] All lesions were excised; *M ulcerans* infection was confirmed by PCR in 21 cases. In ten patients who were treated for 2 weeks or less, viable bacilli could be isolated from excised tissues, whereas *M ulcerans* could not be cultured from tissue taken from 11 patients who were treated for 4 weeks or longer. Lesions either reduced or stabilised in size in all patients.[25] On the basis of these findings, preliminary guidelines were issued by WHO recommending streptomycin in combination with rifampicin as standard treatment for *M ulcerans* infection, with or without additional surgical debridement or skin grafting.[26] When our study was designed, clarithromycin was believed to have only bacteriostatic activity in vivo.[3,27]

We assessed the efficacy of antibiotic therapy with oral rifampicin and intramuscular streptomycin given for 8 weeks for treatment of early *M ulcerans* infection in patients from Ghana. This regimen was compared with rifampicin and streptomycin given for 4 weeks, followed by an oral combination of clarithromycin and rifampicin for 4 weeks. Our aim was to identify an effective alternative treatment to extensive surgical debridement, and to explore possibilities to keep the use of injectable antimicrobial treatment to a minimum.

Methods

Participants

The study design was partly based on discussions within a WHO expert group on Buruli ulcer that took place between 2001, and 2003. Between April, 2006, and January, 2008, patients were recruited at two sites (Nkawie-Toase Government Hospital, Nkawie, and Agogo Presbyterian Hospital, Agogo) in Ghana. Patients clinically

diagnosed with *M ulcerans* disease were recruited by active case finding. Patients were eligible for enrolment if they were aged 5 years or older, had a reported disease duration of less than 6 months, and had lesions with a cross-sectional diameter (indurated area) of 10 cm or less. *M ulcerans* infection was confirmed by IS2404 dry-reagent- based PCR.[28] Exclusion criteria were pregnancy, drug in-tolerance, and renal, hepatic, and acoustic impairment.

The protocol and consent forms were approved by the Committee on Human Research, Publication, and Ethics of the School of Medical Science, Kwame Nkrumah University of Science and Technology, Kumasi, and the Komfo Anokye Teaching Hospital, Kumasi (CHRPE/07/01/05), and by the Ethical Review Committee of Ghana Health Services (GHS-ERC-01/01/06). The Medical Ethics Review Committee of the University Medical Centre Groningen, Netherlands, reviewed the protocol before ethics clearance in Ghana. Written and verbal informed consent was obtained from all participants aged 12 years or older, and from parents, carers, or legal representatives of participants aged 18 years or younger.

Procedures

After participants had given informed consent, we obtained demographic and clinical information and took blood samples. We undertook pregnancy tests in female participants aged 10 years or older, and hearing tests in all participants (AS208 portable equipment; Interacoustics, Assens, Denmark) to obtain baseline audiometric data. HIV antibody testing was done with cold-stored sera after completion of the study. Lesions were photographed and traced onto acetate sheets. Three 3 mm punch biopsy samples were taken under local anaesthesia; two swabs of ulcerated lesions were also taken. All samples were transported to the Kumasi Centre for Collaborative Research in Tropical Medicine laboratory in Kumasi, Ghana, for IS2404 dry-reagent-based PCR and Ziehl-Neelsen staining to detect acid-fast bacilli; mycobacterial culture was done on Löwenstein-Jensen slopes at 32°C.[13] One punch biopsy was reserved for histopathological examination.

Participants started streptomycin (15 mg/kg once daily intramuscularly) and rifampicin (10 mg/kg once daily orally) after the diagnostic procedures. After assessments and start of treatment at the hospital, most participants were treated as outpatients. Once a week, participants were given study drugs to take to the nearest health facility to receive directly observed treatment (DOT) for the subsequent days, with daily wound care. Only participants that had extensive oedema or lesions at difficult sites (joints, eye, or genitalia), or lesions with suspected secondary infection were admitted to hospital; participants who could not receive DOT or wound care at

home were also admitted to hospital. DOT was recorded on forms by the health-care worker or helper who was observing the treatment. Participants were followed up at weekly intervals during the first 8 weeks. At these visits, clinical assessments and digital photographs were taken, DOT forms were checked, and participants were invited to report any adverse events. Once every 2 weeks, the size of the lesion was traced onto an acetate sheet and blood cell counts were taken; we also undertook liver and kidney function tests and hearing tests in all participants, and pregnancy tests in female participants aged 10 years or older.

Randomisation and masking

Before the end of week 4, participants with *M ulcerans* infection confirmed by PCR were randomly assigned to receive streptomycin intramuscularly and rifampicin orally for 4 more weeks (8-week streptomycin group) or rifampicin and clarithromycin (7·5 mg/kg once daily), both orally, for another 4 weeks (4-week streptomycin plus 4-week clarithromycin group). Randomisation was done with minimisation for study site and type of lesion (ulceration or no ulceration). The study coordinator (WAN) forwarded the information of every enrolled participant by mobile telephone text messaging to a statistician (JPS) at the Department of Epidemiology, University Medical Centre Groningen, Netherlands. There, a computer-generated randomisation program was used, and the randomly assigned allocation was then sent by text message to the study coordinator. Individuals who were clinically diagnosed with M ulcerans disease but who did not have confirmation by PCR continued treatment with streptomycin plus rifampicin and were not randomised; these individuals were followed up and analysed separately. This was an open-label trial.

Follow-up and study outcomes

After 8 weeks of antimicrobial treatment, missed doses were not supplemented. Participants were followed up at week 10 and week 12 after start of treatment, and then monthly to week 36, and bimonthly to week 52. Study visits included clinical assessment with reporting of adverse effects, measurement of lesion size (if not healed) by tracing onto an acetate sheet, and photography of the lesion. Participants' travel costs were reimbursed and small monthly incentives (sugar, condensed milk, and cocoa powder) were offered for time spent in the study.

Treatment failure was recorded if a participant's lesion had not healed by week 52, lesion recurrence occurred within 1 year, or lesion size increased to 150% or more at any timepoint compared with baseline with surgical debridement undertaken as deemed necessary by the attending doctor in the hospital. The investigators who

took measurements of the lesions were not masked to treatment assignment. The attending doctor in the hospital making the final decision for extensive surgical debridement was unaware of treatment allocation. Removal of necrosis and slough is part of normal wound care and skin grafting speeds up heeling but does not affect bacterial load. These interventions were therefore not regarded as evidence of treatment failure.

The primary clinical endpoint was lesion healing (complete re-epithelialisation) at 1 year after the start of treatment without recurrence or extensive surgical debridement. Secondary outcomes were time to wound healing and time to complete wound coverage by a crust. Daily sterile dressings were only applied at the health facility if lesions were open and discharging.

Before final healing occurs, lesions might turn dry with a crust. At this stage, participants could cover the lesions for protection at home, without visiting the health facility to receive wound care and sterile dressings. Since participants reported this stage of wound healing as beneficial, we also measured time to complete wound coverage by a crust without complete re-epithelialisation as a secondary endpoint. The safety outcome measure was occurrence of adverse events.

Statistical analysis

When the study was designed, there was no information available about healing rates for the proposed regimens; therefore, we assumed a healing rate of 80% in the 8-week streptomycin group. We calculated that a sample size of 148 randomised and fully assessable participants (74 in each group) would be needed to detect a difference in healing rate of 20% or more (<60% in the 4-week streptomycin plus 4-week clarithromycin group) with a one-sided alpha of 0·05 and a power of 80%.

We calculated an odds ratio for the primary clinical endpoint by use of Fisher's exact test. Because secondary outcome data were interval-censored, we analysed the cumulative incidence of healing by use of actuarial life table analysis and weighted log-rank tests for interval-censored data, in particular the group proportional hazards model[29] and a generalised Wilcoxon-Mann-Whitney test,[30] which emphasises early events. We calculated the exact permutation p value for the scores of the group proportional hazards model and Wilcoxon-Mann-Whitney tests and the non-parametric maximum likelihood estimate of the survival distribution function.[31] Other secondary outcome measures were assessed by actuarial life table analysis. All analyses were by intention to treat. Statistical analysis was done with SPSS version 16.0, R version 0.7–5.5, and Stata version 10.1.

An independent data safety monitoring board reviewed the data for safety purposes

after inclusion of 57 and 115 participants. Interim reports were discussed at the annual WHO meeting on Buruli ulcer in Geneva in 2007 and 2008, and presented at the 2008 combined ICAAC/IDSA Annual Meeting in Washington, DC, USA.[32] After the trial had been completed, two independent wound experts from University Medical Centre Groningen, who were masked to treatment assignment, assessed the primary study endpoint (healing at 1 year) using the digital photographs of the lesions taken during the trial. This trial is registered with ClinicalTrials.gov, number NCT00321178.

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report, or in decisions about submission of results for publication. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Figure 1 shows the trial profile. 180 eligible patients started treatment. 26 patients with suspected but unconfirmed *M ulcerans* infection received streptomycin and rifampicin for 8 weeks. Of 151 participants who were enrolled and randomised, eight had a clinical diagnosis without confirmation of *M ulcerans* infection by PCR. Five of these eight participants had infection later confirmed by one or more diagnostic tests (Ziehl-Neelsen staining, two; culture, one; histopathology, two). Three randomised participants did not have diagnosis confirmed by any test. Table 1 shows baseline characteristics of study participants. Lesions were more frequently seen on the right side of the body (64%) than on the left side (36%; $p<0{\cdot}0001$). Three (2%) participants were HIV positive; these individuals had initial lesions and clinical presentations that were indiscernible from those of HIV-negative participants.

One participant in the 8-week streptomycin group withdrew from the study at week 6. In the 4-week streptomycin plus 4-week clarithromycin group, two participants moved out of the study area and were lost to follow-up (week 32 and 36) and one participant, who later tested positive for HIV infection, died in week 16 of urosepsis. Since these four participants had healed lesions at their last assessment, they were included in the analysis for the primary clinical endpoint and in the analyses for time to healing.

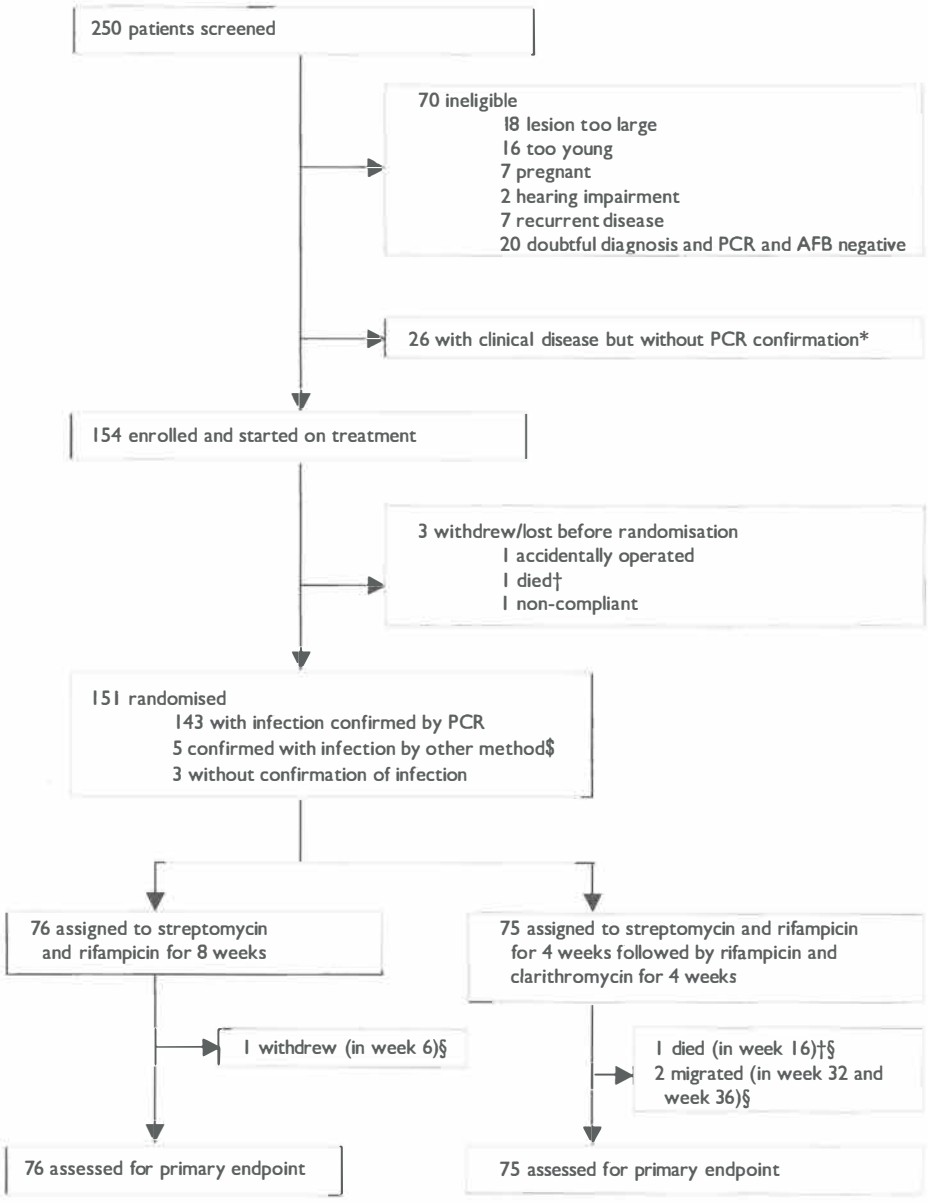

Figure 1: Trial profile

AFB=acid-fast bacilli. *Patients not enrolled but given 8 weeks of treatment with streptomycin and rifampicin. †Participant died of cause unrelated to *M ulcerans* infection. $See text for details. §Healed at time of last assessment, included in the final analysis.

Table 1: Patient baseline characteristics

	8-week streptomycin group (n=76)	4-week streptomycin plus 4-week clarithromycin group (n=75)
Sex (male)	27 (36%)	19 (25%)
Age (years)	12 (8-18)	12 (9-22)
Body-mass index (kg/m^2)	16.6 (14.9-19.4)	17.2 (15.4-19.4)
Study site		
Agogo	54 (71%)	53 (71%)
Nkawie	22 (29%)	22 (29%)
Tribe		
Akan	20 (26%)	18 (24%)
Other	56 (74%)	57 (76%)
Duration of disease (weeks)	4 (2-6)	3 (2-4)
Lesion surface area (cm^2)	29 (9-55)	26 (10-46)
Category of lesion		
I	29 (38%)	29 (39%)
II or III	47 (62%)	46 (61%)
Type of lesion		
No ulceration	49 (64%)	43 (57%)
Ulceration	27 (36%)	32 (43%)
Lesion distribution (side of body)		
Left	27 (36%)	28 (37%)
Right	49 (64%)	47 (63%)
HIV infection	0 (0%)	3 (4%)

Data are n (%) or median (IQR). Patients in the 8-week streptomycin group were assigned to receive intramuscular streptomycin and oral rifampicin for 8 weeks. Patients in the 4-week streptomycin plus 4-week clarithromycin group were assigned to receive streptomycin and rifampicin for 4 weeks followed by rifampicin and clarithromycin, both orally, for 4 weeks.

Compliance to study treatment was assessed by use of DOT forms, signed by health personnel at the health facilities. Compliance was 98% in the 8-week streptomycin group and 99% in the 4-week streptomycin plus 4-week clarithromycin group.

Treatment failure was recorded in ten participants, three in the 8-week streptomycin group and seven in the 4-week streptomycin plus 4-week clarithromycin group. Table 2 shows the characteristics of these individuals. Five participants were not healed at week 52, all of whom had a substantial decrease in lesion size. One participant had several lesions, and four had large lesions at the start of treatment (one of whom had HIV infection). Of the five participants with treatment failure before week 52, two had large lesions, one had a pre-ulcerative lesion that ulcerated later, one had a progressive lesion, and one had a lesion that almost healed, but opened up again. No participants with healed lesions had a recurrence at week 52.

Table 2: Characteristics of ten participants with treatment failure

	Study site	Treatment group	Sex	Age (years)	Category of lesion	Stage	Size of lesion (mm)	Additional information	Time point (weeks*)	Additional diagnostics (timepoint, weeks*)	Diagnostic results†
Participants with treatment failure recorded before week 52											
1	Agogo	8-week streptomycin	Male	6	II	Ulcer	120x98	Extensive debridement	6	"	"
2	Agogo	8-week streptomycin	Male	10	III	Ulcer and oedema, critical site	126x79	Extensive debridement	20	"	"
3	Agogo	4-week streptomycin plus 4-week clarithromycin	Female	12	II	Plaque	73x60	Lesion progression, extensive debridement	8	Surgical resected tissue	PCR negative, ZN negative, culture positive
4	Nkawie	4-week streptomycin plus 4-week clarithromycin	Female	11	I	Nodule	30x24	Ulceration of lesion; 4 additional weeks of streptomycin and rifampicin	12	Punch biopsy and swab	PCR negative, ZN negative, culture negative
5	Nkawie	4-week streptomycin plus 4-week clarithromycin	Female	5	I	Plaque	48x47	New ulceration after initial closure	34	Swab and surgical resected tissue	PCR negative, ZN negative, culture negative
Participants who were not healed at time of primary endpoint (week 52)											
6	Agogo	8-week streptomycin	Male	22	II (with oedema leading to III)	Ulcer	112x80	"	"	"	"

7	Agogo	4-week streptomycin plus 4-week clarithromycin	Female	20	III	Ulcer and multiple nodules	"	Ulcer healed; multiple nodules with ulceration before healing	"	Swab (32), swab (52)	32 weeks: PCR positive, ZN negative, culture positive; 52 weeks: PCR positive, ZN positive, culture negative
8	Nkawie	4-week streptomycin plus 4-week clarithromycin	Male	12	II	Ulcer	95x95	Inadequate wound care	"	Swab (29), swab (72), surgically resected tissue (80)	29 weeks: PCR negative, ZN negative, culture negative; 72 weeks : PCR negative, ZN negative, culture positive; 80 weeks: PCR negative, ZN negative, culture negative;
9	Nkawie	4-week streptomycin plus 4-week clarithromycin	Female	7	II	Ulcer	122x100	"	"	"	"
10	Agogo	4-week streptomycin plus 4-week clarithromycin	Female	27	III	Plaque and two small ulcers	113x86	HIV positive	"	"	"

Treatment failure was recorded if a participant's lesion had not healed by week 52, lesion recurrence occurred within 1 year, or lesion size increased to 150% or more at any timepoint compared with baseline with surgical debridement undertaken as deemed necessary by the attending doctor in the hospital. *Weeks after start of treatment. †Results for insertion sequence 2404-dry reagent based PCR, Ziehl-Neelsen (ZN) staining to indentify acid-fast bacilli, and *M ulceran*s culture. Patients in the 8-week streptomycin group were assigned to receive intramuscular streptomycin and oral rifampicin for 8 weeks. Patients in the 4-week streptomycin plus 4-week clarithromycin group were assigned to receive streptomycin and rifampicin for 4 weeks followed by rifampicin and clarithromycin, both orally, for 4 weeks.

73 (96%) participants in the 8-week streptomycin group and 68 (91%) participants in the 4-week streptomycin plus 4-week clarithromycin group had healed lesions at week 52 (odds ratio for failure in healing for 4-week streptomycin plus 4-week clarithromycin vs 8-week streptomycin 2·49, 95% CI 0·66 to infinity, p=0·16). We obtained consistent findings when the four participants who were not followed up to week 52 were excluded from the analysis and when two wound experts masked to treatment assignment assessed the primary endpoint by use of photographs available at the different timepoints (data not shown).

Table 3 shows the actuarial life table for cumulative proportion of healing. The estimated cumulative proportion of patients healed at week 52 was 0·99 (95% CI 0·94–1·00) in the 8-week streptomycin group and 0·96 (95% CI 0·88–0·99) in the 4-week streptomycin plus 4-week clarithromycin group; a difference of 0·034 (95% CI –0·024 to 0·091) between groups.

Figure 2 shows the non-parametric maximum likelihood estimates for healing in the intention-to-treat population. Neither the group proportional hazards model (p=0·26; 99% CI 0·22–0·29) nor the generalised Wilcoxon-Mann-Whitney test (p=0·60; 99% CI 0·56–0·64) showed a significant difference in time to healing between groups. The group proportional hazards model suggested a shorter time to healing in the 8-week streptomycin group whereas the Wilcoxon-Mann-Whitney test suggested that time to healing was shorter in the 4-week streptomycin plus 4-week clarithromycin group. Adjustment for study site and type of lesion (ulceration or no ulceration) did not affect the results (data not shown).

Five participants received skin grafts, four in the 8-week streptomycin group (at week 16, 24, 24, and 28), and one in the 4-week streptomycin plus 4-week clarithromycin group (at week 20). Time to healing of category I lesions (median 18 weeks, 95% CI 14–22) was significantly shorter than that for category II and III lesions (30 weeks, 95% CI 26–34, p=0·002; data pooled for the two treatment groups; five participants with skin grafts not included). Time to complete wound coverage by a crust was also significantly shorter for category I lesions than category II and III lesions (14 weeks, 95% CI 11–18, vs 22 weeks, 95% CI 22–26; p=0·002).

Three participants had vestibulotoxic events, one in the 8-week streptomycin group (aged 49 years, starting after 7 weeks of treatment) and two in the 4-week streptomycin plus 4-week clarithromycin group (aged 24 years and 38 years, starting after 4 weeks and 3 weeks of treatment, respectively). Analysis of digital photographs showed that three participants had mild to moderate functional limitations at the end of the study: one had a contracture with substantial decrease in range of movement of the thumb and index finger (4-week streptomycin plus 4-week clarithromycin

group); two had ulcers on the back of the hand and wrist that resulted in claw-hands (one in each group). No liver or kidney function test abnormalities or audiological deterioration occurred that necessitated termination of streptomycin treatment. One participant developed an injection abscess (4-week streptomycin plus 4-week clarithromycin group) and two participants (both in the 4-week streptomycin plus 4-week clarithromycin group) developed an abscess close to the initial lesion which was incised and drained. One participant in the 8-week streptomycin group and two participants in the 4-week streptomycin plus 4-week clarithromycin group reported abdominal discomfort.

Table 3: Actuarial life table for cumulative proportion of healing for both treatment groups, by time interval

	8-week streptomycin group					4-week streptomycin plus 4-week clarithromycin group				
	Total (n)	Healed (n)	Treatment failure (n)	Cumulative proportion healed	95% CI	Total (n)	Healed (n)	Treatment failure (n)	Cumulative proportion healed	95% CI
Week 1	76	1	0	0.013	0.0019–0.090	75	0	0		
Week 2	75	3	0	0.053	0.020–0.13	75	1	0	0.013	0.019–0.091
Week 4	72	1	0	0.066	0.028–0.15	74	1	0	0.027	0.0067–0.10
Week 5	71	1	0	0.076	0.036–0.17	73	1	0	0.040	0.013–0.12
Week 6	70	0	1	0.076	0.036–0.17	72	4	0	0.093	0.046–0.19
Week 7	70	0	0	0.076	0.036–0.17	68	2	0	0.12	0.064–0.22
Week 8	69	2	0	0.11	0.054–0.20	66	2	1	0.15	0.084–0.25
Week 8-10	67	1	0	0.12	0.064–0.22	63	2	1	0.17	0.11–0.28
Weeks 10-12	66	3	0	0.16	0.094–0.26	60	5	0	0.24	0.16–0.36
Weeks 12-16	63	4	0	0.21	0.14–0.32	55	5	0	0.32	0.22–0.43
Weeks 16-20	59	12	1	0.37	0.28–0.49	50	10	0	0.45	0.34–0.57
Weeks 20-24	46	9	0	0.50	0.39–0.61	40	5	0	0.52	0.41–0.64
Weeks 24-28	37	10	0	0.63	0.52–0.74	35	7	0	0.61	0.51–0.73
Weeks 28-32	27	9	0	0.76	0.65–0.85	28	7	0	0.71	0.60–0.81
Weeks 32-36	18	11	0	0.90	0.82–0.96	21	11	1	0.87	0.78–0.93
Weeks 36-44	7	4	0	0.96	0.90–0.99	9	2	0	0.90	0.81–0.95
Weeks 44-52	3	2	1	0.99	0.94–1.00	7	3	4	0.96	0.88–0.99

Patients in the 8-week streptomycin group were assigned to receive intramuscular streptomycin and oral rifampicin for 8 weeks. Patients in the 4-week streptomycin plus 4-week clarithromycin group were assigned to receive streptomycin and rifampicin for 4 weeks followed by rifampicin and clarithromycin, both orally, for 4 weeks.

Table 4: *M ulcerans* isolated by culture of tissue specimens after 8-week treatment period

	Study site	Sex	Age (years)	Category of lesion	Stage	Indication for culture	Timepoint (weeks)*	Additional information	Diagnostic specimen
Patient 1	Agogo	Female	12	II	Plaque	Lesion progression	11	Extensive surgical debridement	Surgically resected tissue
Patient 2	Nkawie	Female	12	II	Oedema	Development of a second lesion	14	Lesion healed without further intervention	Swab
Patient 3	Nkawie	Female	5	II	Ulcer	Pus collection	18	Lesion healed without further intervention	Swab
Patient 4	Agogo	Female	20	III	Ulcer and multiple nodules	Confirmation of nodule	32	Ulcer healed; multiple nodules ulcerated before healing†	Swab
Patient 5	Nkawie	Male	12	II	Ulcer	No complete healing at week 52	72	Inadequate wound care; surgical debridement	Swab

This analysis was not specified by the protocol. All five participants were in the 4-week streptomycin plus 4-week clarithromycin group (streptomycin and rifampicin for 4 weeks followed by rifampicin and clarithromycin for 4 weeks). *Time of tissue specimen collection (weeks after start of treatment). †Participant with treatment failure.

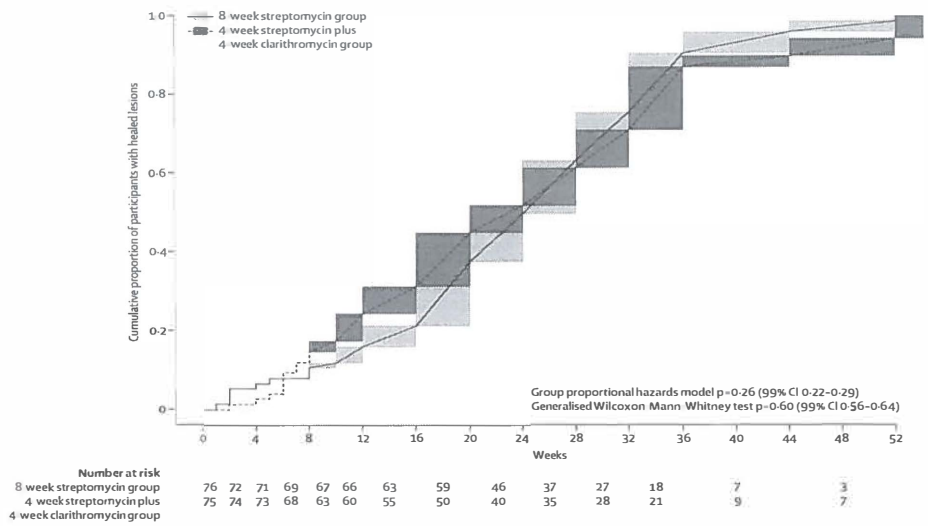

Figure 2: Non-parametric maximum likelihood estimates for time to healing.
The non-parametric maximum likelihood estimates for each treatment group are plotted with shaded rectangles denoting the indeterminate rises in the proportion healed during each time interval. Linear interpolation lines of healing within these indeterminate regions are also shown. Patients in the 8-week streptomycin group were assigned to receive intramuscular streptomycin and oral rifampicin for 8 weeks. Patients in the 4-week streptomycin plus 4-week clarithromycin group were assigned to receive streptomycin and rifampicin for 4 weeks followed by rifampicin and clarithromycin, both orally, for 4 weeks.

Some participants had additional diagnostic tests not specified in the protocol. Table 4 shows the characteristics of the five participants in whom *M ulcerans* was isolated by culture after treatment; all were in the 4-week streptomycin plus 4-week clarithromycin group. Three of these five participants had treatment failure: in two, surgical debridement was done; in the third, multiple nodules ulcerated successively over 52 weeks before final healing. Two participants had lesion healing without further intervention within the study period.

Discussion

Our study has shown that early, limited *M ulcerans* infection can be safely and effectively managed by antimicrobial treatment alone, without surgical debridement. The drug regimen proposed by WHO, consisting of 8 weeks of streptomycin and rifampicin, seemed effective and was not associated with deterioration requiring subsequent surgical debridement. Treatment with oral clarithromycin plus rifampicin

during the second 4-week period resulted in similar outcomes to continuation of treatment with streptomycin and rifampicin. Our findings are important for patients with *M ulcerans* infection who live in remote, resource-poor areas in West Africa, where people often need to walk for several hours to reach health-care facilities, skilled personnel are scarce, and patients tend to refrain from treatment because of fear of surgery. Our results also support the use of antimicrobial treatment in individuals who are unable to receive streptomycin—eg, pregnant women or those who cannot tolerate aminoglycosides. With few reported side-effects, the treatment regimens used in this trial seemed well tolerated, although vestibulotoxicity remains a concern. The rate of lesion recurrence in our study at 52 weeks was lower than that reported in retrospective studies assessing the effect of surgery, in which rates of between 6% and 47% were reported.[20–22]

Time to healing was a median of 18 weeks for category I lesions and 30 weeks for larger lesions. The length of this healing period might have obscured the potential of antimicrobial treatment in earlier studies that either looked at healing after 2 months,[24] or assigned participants to surgery when early healing was not seen during follow-up.[23] HIV was not an important confounder; most case-control studies from West Africa report a low incidence of HIV in patients with *M ulcerans* infection.[33,34]

One strength of this study is the large proportion of participants (147 of 151) who were followed up to week 52. Second, most participants (148 of 151) had laboratory-confirmed *M ulcerans* infection, which contrasts with previous trials that were partly undertaken before PCR-based diagnostic confirmation tests were available.[23,24] Finally, the sample size in our study was substantially larger than that in earlier studies.

A potential weakness of our study is the open-label design. However, masking would have substantially increased costs, and a trial in which children can be assigned to placebo injections is not justified for safety reasons. Moreover, although only one injection abscess was recorded, intramuscular injections in rural Africa are not the preferred option. Another limitation of our study is that no formal external monitoring was done. However, limited auditing was organised. Additionally, consistent results were obtained when two wound experts who were masked to treatment assignment reviewed all digital photographs available at the different timepoints. We therefore believe that the study was robust.

One concern is that healing took a fairly long time. Additionally, we could not address the issue of prevention of disabilities in a formal way, although our analysis of digital photographs combined with clinical assessment showed that only three participants had mild to moderate functional limitations at week 52 (all three involving hand function). Contractures and functional limitations are common in ulcers that are

close to joints.[11] Future studies should assess prevention of disabilities, include all categories of lesions, and investigate oral drug regimens.

Thus, antimicrobial treatment is highly effective for treatment of early, limited *M ulcerans* infection, and the number of intramuscular injections of streptomycin can be reduced without compromising efficacy.

Contributors

TSvdW and YS designed and supervised the study. WAN coordinated the study. WAN, WAT, PCA, and EOA were responsible for patient screening and enrolment. KMA, WT, and WAN provided patient care and requested informed consent from participants, participants' parents, or legal representatives, and collected the clinical and laboratory data. GB, VS, NYA-B, and OA were responsible for the laboratory confirmation. JPS, WAN, and YS did the statistical analyses. TSvdW, WAN, and YS contributed to the interpretation of the results and the writing and critical review of the report. All authors have seen and approved the final version of the report.

Conflicts of interest

We declare that we have no conflicts of interest.

Acknowledgments

We thank all study participants and health-care workers involved in the trial. We thank Richard O Phillips for invaluable help during the start of the trial, Cari J Stek for occasional monitoring activities, the "de Sprong" outpatient pharmacy team, University Medical Centre Groningen for drug supply, Marieke M de Waard and Janneke Huizinga for undertaking masked assessment of digital images of the participants' lesions, William R Faber, Paul D R Johnson, and Alan J Knell for serving on the data safety monitoring board, and Kingsley Asiedu, Global Buruli Ulcer Initiative, WHO, Geneva, Switzerland. This study was supported by the European Union (EU FP6 2003-INCO-Dev2-015476) and Buruli Ulcer Groningen Foundation. YS received a ZonMW AGIKO grant (Netherlands Foundation for Research). WAN is currently at the Department of Internal Medicine, Medical Center Leeuwarden, Leeuwarden, Netherlands. YS is currently at Department of Internal Medicine, Division of infectious Diseases, Tropical Medicine and AIDS, Academic Medical Centre, University of Amsterdam, Amsterdam, Netherlands.

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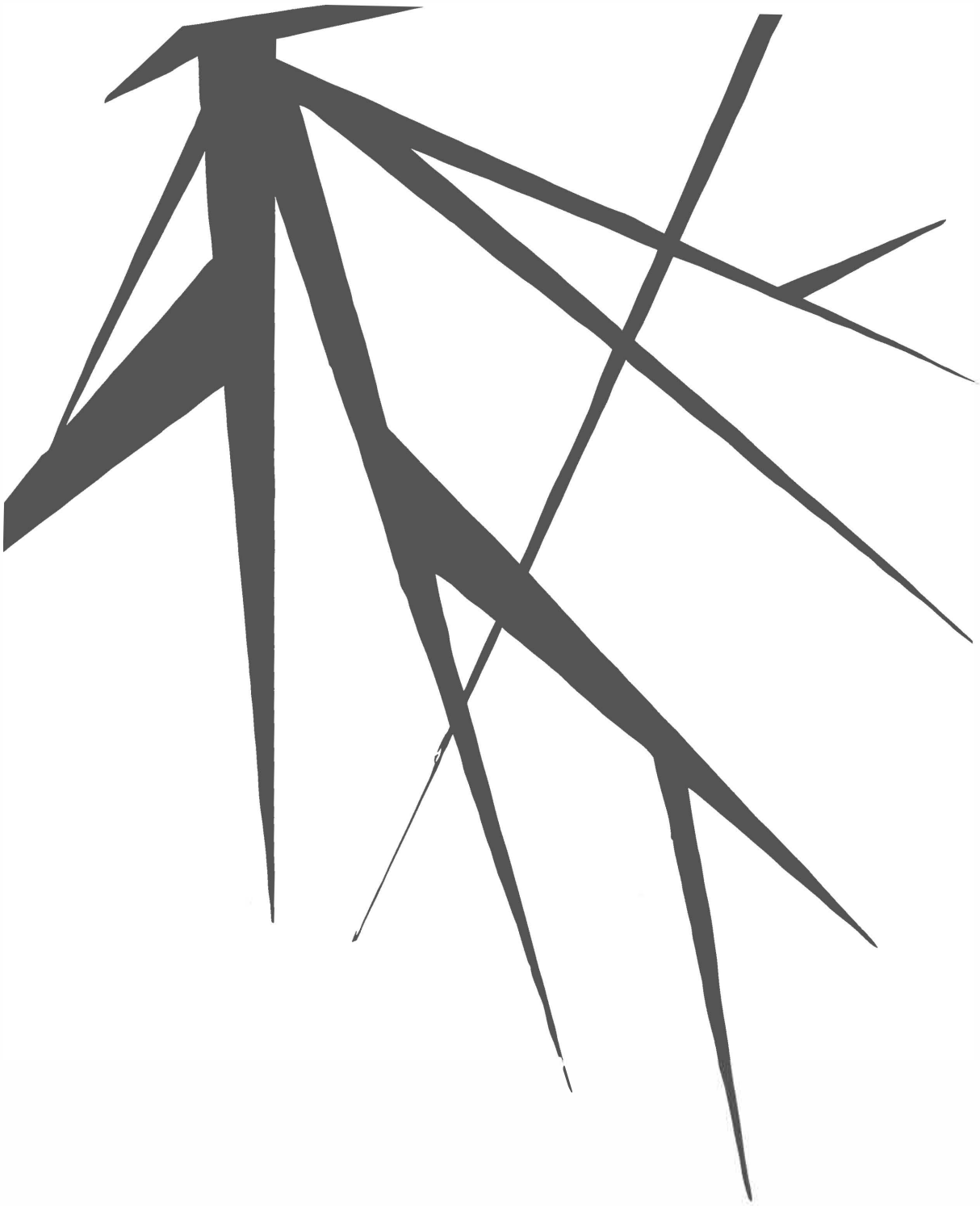

CHAPTER 3.1

Pharmacokinetics of rifampin and clarithromycin in patients treated for *Mycobacterium ulcerans* infection

J.W. C.Alffenaar
W.A. Nienhuis
F. de Velde
A.T. Zuur
A. M.A.Wessels
D.Almeida
J. Grosset
O.Adjei
D. R.A. Uges
T. S. van der Werf

Antimicrob Agents Chemother. 2010 Sep;54(9):3878-83

Summary

In a randomized controlled trial in Ghana, treatment of *Mycobacterium ulcerans* infection with streptomycin (SM)-rifampin (RIF) for 8 weeks was compared with treatment with SM-RIF for 4 weeks followed by treatment with RIF-clarithromycin (CLA) for 4 weeks. The extent of the interaction of RIF and CLA combined on the pharmacokinetics of the two compounds is unknown in this population and was therefore studied in a subset of patients. Patients received CLA at a dose of 7.5 mg/kg of body weight once daily, rounded to the nearest 125 mg. RIF was administered at a dose of 10 mg/kg, rounded to the nearest 150 mg. SM was given at a dose of 15 mg/kg once daily as an intramuscular injection. Plasma samples were drawn at steady state and analyzed by liquid chromatography-tandem mass spectroscopy. Pharmacokinetic parameters were calculated with the MW/Pharm (version 3.60) program. Comedication with CLA resulted in a 60% statistically nonsignificant increase in the area under the plasma concentration-time curve (AUC) for RIF of 25.9 mg • h/liter (interquartile ratio [IQR], 21.9 to 31.5 mg • h/liter), whereas the AUC of RIF was 16.2 mg • h/liter (IQR, 15.0 to 17.6 mg • h/liter) in patients comedicated with SM ($P = 0.09$). The median AUCs of CLA and 14-hydroxyclarithromycin (14OH-CLA) were 2.9 mg • h/liter (IQR, 1.5 to 3.8 mg • h/liter) and 8.0 mg • h/liter (IQR, 6.7 to 8.6 mg • h/liter), respectively. The median concentration of CLA was above the MIC of *M. ulcerans*, but that of 14OH-CLA was not. In further clinical studies, a dose of CLA of 7.5 mg/kg twice daily should be used (or with an extended-release formulation, 15 mg/kg should be used) to ensure higher levels of exposure to CLA and an increase in the time above the MIC compared to those achieved with the currently used dose of 7.5 mg/kg once daily.

Introduction

Although many antimycobacterial agents appeared to be effective against *Mycobacterium ulcerans* infections in in vitro and in animal models (4, 11, 27, 30), clinical evidence of the effectiveness of antimicrobial treatment was predominantly based on a small study conducted with patients in Ghana (15). Conceivably, using antimycobacterial agents results not only in preventing bacilli from replicating and killing microorganisms but also in halting the production of the toxin mycolactone (36). This toxin that causes the tissue damage is produced by enzymes encoded by the pMUM001 plasmid (33). Current WHO recommendations suggest 8 or more weeks of treatment with rifampin (RIF) plus streptomycin (SM) for all clinical forms of active Buruli ulcer disease (BUD). Daily injections with streptomycin are problematic, as most patients live in remote areas with limited health care facilities. Proper hygiene with these injections, as well as intrinsic ototoxicity and renal toxicity, is a concern. Therefore, an oral treatment schedule is urgently needed to reduce the number of injections and to improve the tolerability and safety of the proposed regimen. Pregnant women might also benefit from treatment without aminoglycosides. This problem was addressed by comparing 8 weeks of SM (15 mg/kg of body weight) and RIF (10 mg/kg) treatment (8SR arm) and 4 weeks of streptomycin and rifampin treatment followed by 4 weeks of RIF plus clarithromycin (CLA; 7.5 mg/kg) treatment (4SR/4CR arm) in a randomized controlled trial (the BURULICO trial) (26). CLA was chosen for inclusion in the treatment regimen because of in vitro data suggesting that this drug is active against *M. ulcerans*, for which the MICs range from 0.125 to 2.0 mg/liter (30). In a pharmacokinetic (PK) study with adults who received doses of 500 mg twice daily, plasma CLA concentrations (5) were well above the MIC for most *M. ulcerans* isolates. The clinical effectiveness of macrolides is only partly explained by pharmacokinetics, because these drugs typically accumulate in inflammatory cells, especially macrophages, at the site of infection (1). Although *M. ulcerans* infection has long been regarded a predominantly extracellular infection (20), evidence has emerged from animal models that *M. ulcerans* infection has an intracellular stage in which it multiplies inside macrophages (10, 34). These data taken together suggest that the intramacrophage CLA concentration might add to the beneficial effect of the drug to fight *M. ulcerans* infection and that CLA might exert its effect inside these immune cells without reaching inhibitory drug concentrations in the bloodstream. On the other hand, if plasma CLA concentrations do not reach inhibiting or mutant-inhibiting concentrations, at least in the extracellular space where bacilli are present during later stages of the disease, inadvertent monotherapy with RIF

alone would result. At the time that the present study was designed, the activity of the 14-hydroxyclarithromycin (14OH-CLA) metabolite against *M. ulcerans* was unknown, and sensitivity to 14OH-CLA therefore had to be determined. In mycobacterial infections, monotherapy has invariably resulted in the failure of treatment, as drug-resistant pathogens within the total microbial load might escape and repopulate the diseased lesions in the host (9). RIF is known to induce cytochrome P450 isoenzymes (e.g., CYP 3A4, involved in the elimination of CLA), while CLA is also known to inhibit the enzyme activity of CYP 3A4 (3, 18). The P-glycoprotein (Pgp) efflux transporter is also affected, as RIF induces and is the substrate of Pgp and CLA inhibits Pgp (7, 14). Earlier studies with patients with *M. avium* infections showed an induction of metabolism of CLA, but the data on 14OH-CLA were not consistent between the two studies (28, 37). The purpose of the present study was to assess the influence of this CLA-RIF interaction on the areas under the plasma concentration-time curves (AUCs) for the 4SR/4CR study arm and to compare those AUCs to the AUC for RIF in patients who were treated in the 8SR study arm. In addition, the average time that plasma drug concentrations were maintained in excess of the MIC was studied.

Materials and methods

The open-label prospective pharmacokinetic study described here evaluated the pharmacokinetics of RIF and RIF combined with CLA in the treatment of *Mycobacterium ulcerans* disease in patients already enrolled in the BURULICO trial. The study was conducted at the Nkawie-Toase Governmental Hospital and at the Agogo Presbyterian Hospital, both in the Ashanti region of Ghana.

Study subjects

Patients ≥10 years of age (male and female) were eligible for inclusion in this side study to assess the pharmacokinetics of clarithromycin and rifampin when they were allocated to the 4SR/4CR arm and the pharmacokinetics of rifampin when they were allocated to the 8SR arm. Exclusion criteria for the BURULICO trial were treatment with macrolide or quinolone antibiotics, antituberculosis medication, or immunomodulatory drugs (including corticosteroids) within the previous 1 month; current treatment with any drugs likely to interact with the study medication, e.g., anticoagulants, cyclosporine, phenytoin, oral contraceptives, and phenobarbitone; a history of hypersensitivity to rifampin, streptomycin, and/or clarithromycin; and an

inability to take oral medication or the presence of a gastrointestinal disease likely to interfere with drug absorption.

Drug administration

Patients received CLA at a dose of 7.5 mg/kg once daily, which was rounded to the nearest 125 mg. RIF was administered at a dose of 10 mg/kg, which was rounded to the nearest 150 mg. The drugs were administered on an empty stomach, but the participants were allowed to take a light standardized breakfast after drug ingestion. Although food intake does not influence the AUC of CLA (8), it influences the AUC of RIF (39). By offering a standardized light breakfast approximately 30 min after drug ingestion, the effect on drug absorption would be minimized and equally distributed over both groups. The adherence to the treatment regimen was 100%, as the participants were in a guided patient program.

Pharmacokinetic assessment

On the day of this PK study, a full pharmacokinetic curve was recorded at steady state after a minimum of 7 days of consecutive treatment with the same dose. Blood samples (2 ml, EDTA anticoagulant) were obtained before a dose of CLA-RIF or RIF was administered (time zero) and at 0.5, 1, 1.5, 2, 2.5, 3, 5, 7.5, and 10 h after administration of the dose. This schedule was based on earlier data (17, 29), with the restriction that the participants in the present study had to be able to return to their homes (often, in remote rural villages) on the evening of the sampling day. Plasma was separated and frozen at 20°C until it was processed.

Analytical methods

The plasma concentrations of RIF and 25-desacetylrifampicin (25DA-RIF) and of CLA and 14OH-CLA were determined at the Laboratory for Clinical Toxicology and Drugs Analysis of the Department of Hospital and Clinical Pharmacy of the University Medical Center Groningen, Groningen, Netherlands, using a validated liquid chromatography-tandem mass spectrometry (LC/MS/MS) assay (13).

Pharmacokinetic analysis

The values of the pharmacokinetic parameters were calculated using the KINFIT program (MW/Pharm, version 3.60; Mediware, Netherlands) (31). C_{max} was defined as the highest observed serum concentration, and T_{max} was the time corresponding to C_{max}. C_{min} was the serum concentration before intake of the dose. The AUC from time zero to 24 h (AUC_{0-24}) was calculated using the log-linear trapezoidal rule.

The elimination half-life ($t_{1/2}$) was calculated by $0.693/k_{el}$. The apparent clearance of the drug (CL/*F*) was calculated as the dose/AUC_{0-24}, and the apparent volume of distribution (*V*/*F*) was calculated as the dose/concentration at steady state.
For both treatment groups, a population one-compartment model with first-order absorption pharmacokinetics with lag time was generated using the RIF dose, the body surface area of the participants, and the observed RIF serum concentrations in an iterative two-stage Bayesian procedure (31). Pharmacokinetic parameters were assumed to be log-normally distributed. Residual error was assumed to be distributed normally with a standard deviation (SD) of 0.1 + (0.25 x *C*), where *C* is the serum rifampin concentration. Since the participants received RIF only orally, bioavailability could not be assessed and was fixed at a value of 1, as the bioavailability of RIF is nearly complete.

Bacteriologic assessment

The MICs of CLA and 14OH-CLA were determined by the agar proportion method on Middlebrook 7H11 agar at pH 6.6 supplemented with 10% oleic acid-albumin-dextrose-catalase. For both drugs, 2-fold concentrations ranging from 0.125 to 8 mg/ml were tested using two different strains of *M. ulcerans*: strain 1059, a recent human isolate from Ghana (38), and strain 1615 (ATCC 35840), a well-characterized Malaysian human isolate (16). The strains were subsequently passaged in mice in a laboratory at Johns Hopkins University School of Medicine. These reference strains instead of test isolates from our patients were used, as isolation of *M. ulcerans* by culture has a very low yield (21). Bacillary suspensions adjusted to an optical density at 600 nm of 1.0 were diluted to 10^{-5} in phosphate-buffered saline; and 0.5 ml of each of the 10^{-1}, 10^{-3}, and 10^{-5} dilutions was inoculated onto plates with and without drugs. The plates were then incubated at 32°C, and the colony counts were read 2 and 3 months later. The MIC was defined as the lowest drug concentration inhibiting ≥99% of the colony counts on the control plates.

Statistical methods

Data are presented as median values and interquartile ranges (IQRs). Differences in age, body mass index, and the values of the pharmacokinetic parameters between the patients groups were assessed with the Wilcoxon rank-sum test (Mann-Whitney U test) for unpaired data. A sample size of 10 was estimated to be needed to detect peak CLA concentrations of >0.5 mg/liter in 80% of the patients with a statistical power of 80% and a significance level of 5%. A sample size of five was estimated to be needed to detect a 20% increase in the RIF concentrations in the presence of CLA

compared to the RIF concentrations in the group treated with RIF and streptomycin with a statistical power of 80% and a significance level of 5%.

Ethics

The present study was a side study of a randomized trial (ClinicalTrials.gov identifier NCT00321178). As for the main study protocol, written and verbal informed consent was obtained from participants of 10 years and over and from their custodians if they were below 18 years of age. The protocol for the present study was also approved by the local institutional ethics committees of the Kwame Nkrumah University of Science and Technology and the Komfo Anokye Teaching Hospital. Only individuals over age 10 years were eligible for participation in the present study. All study participants gave informed consent after being given an appropriate amount of time to consider their participation; a small incentive in cash and kind was offered, and for participants aged between 10 and 18 years, their parents or caretakers also gave written informed consent.

Results

Study subjects

Thirteen patients (12 females and 1 male) were included in the present study: 8 were in the 4SR/4CR arm and 5 were in the 8SR arm. The baseline age, body weight, and body mass index characteristics were similar for the two groups. The patient characteristics are presented in Table 1. Twelve of 13 patients had Buruli ulcer lesions, as confirmed by IS*2404*-based PCR (21). The Buruli ulcer lesions of these 12 patients were completely reepithelialized within the time frame of the study (healing without a recurrence within 1 year after inclusion). The one unconfirmed lesion—a nodule—in a patient treated with 8SR was excised 2 weeks after the end of treatment because another diagnosis was suspected. After excision, that patient also tested IS*2404*-based PCR positive.

Bacteriologic assessment

The MICs of CLA were 0.25 mg/liter and 0.50 mg/liter for *M. ulcerans* 1059 and *M. ulcerans* 1615, respectively. The MICs of 14OH-CLA were 4 mg/liter and 8 mg/liter for *M. ulcerans* 1059 and *M. ulcerans* 1615, respectively. For both strains, the MIC of 14OH-CLA was 16 times higher than that of CLA.

Pharmacokinetic study

The patients in both study arms received the same dose of RIF per kg of body weight. The patients in the 8SR study arm received RIF at a dose of 8.8 mg/kg (IQR, 8.6 to 10.0 mg/kg) once daily, which was not significantly different from the RIF dose of 8.8 mg/kg (IQR, 8.4 to 10.8 mg/kg) administered in the 4SR/4CR study arm (P = 0.70). The patients had received the study medication for a median duration of 50 days (IQR, 29 to 55 days) before blood samples were drawn. The RIF concentration-time curves of both arms are displayed in Figure 1. The CLA concentration-time curve is displayed in Figure 2. The values of the pharmacokinetic parameters of the two treatment arms are summarized in Table 2. Although the difference was not statistically significant (P = 0.09), the median increase in the AUC_{0-24} value for RIF was 60% if the AUC_{0-24} of 25.9 mg • h/liter (IQR, 21.9 to 31.5 mg • h/liter) for the patients in the 4SR/4CR arm was compared to the median AUC_{0-24} value of RIF of 16.2 mg • h/liter (IQR, 15.0 to 17.6 mg • h/liter) for patients in the 8SR arm. 25DA-RIF could be detected in only 5/13 patients. The geometric mean AUC ratio of CLA to 14OH-CLA was 0.33 (range, 0.22 to 0.39). Population pharmacokinetic analysis showed that apparent clearance of RIF was (not significantly) reduced in patients who also received CLA but was due to interpatient variability in both groups (P = 0.17) (Table 3). The median AUC/MIC ratio of RIF was 52 (IQR, 44 to 63) in patients treated with CLA as well, which was 63% higher (P = 0.09) than the median AUC/MIC ratio of 32 (IQR, 30 to 35) in patients treated with RIF and SM.

Table 1: Baseline characteristics

Characteristic	Result for each treatment arm		*P* value
	4SR/4CR (n=8)	8SR (n=5)	
Age (yr)[a]	26.0 (13.5-41.0)	26.5 (19.5-36.3)	0.07
Gender (M/F)	0/8	1/4	0.4
Wt (kg)[a]	53.5 (51.5-56.5)	45.0 (35.0-60.0)	0.4
BMI (kg/m^2)[a]	18.7 (15.2-22.0)	20.5 (19.0-22.9)	0.17

[a]Data are medians (25^{th} to 75^{th} percentiles).

The times the concentrations remained above the MIC of CLA were 300, 210, 180, 285, 300, 30, 300, and 90 min above 0.25 mg/liter and 170, 105, 10, 135, 190, 0, 200, and 0 min above 0.5 mg/liter for each patient. This resulted in median times that the concentration was above the MIC of 0.25 mg/liter and 0.5 mg/liter of 248 min and 120 min, respectively. In two of the patients, the concentration never reached the MIC of 0.5 mg/liter. The concentration of 14OH-CLA was never above MICs of 4 mg/liter and 8 mg/liter.

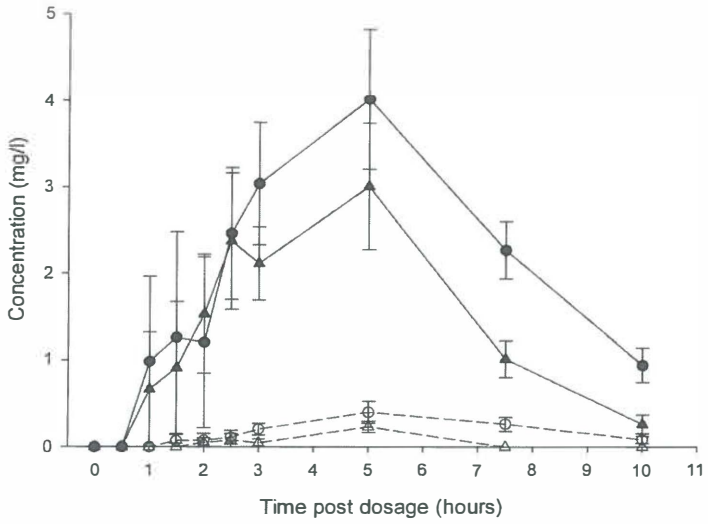

FIG. 1: RIF/25DA-RIF concentration-time curve. Mean (standard error) plasma concentration-time curves for RIF (solid symbols) and 25DA-RIF (open symbols) from the 4SR/CR4 arm (circles) and SR8 arm (triangles) are shown.

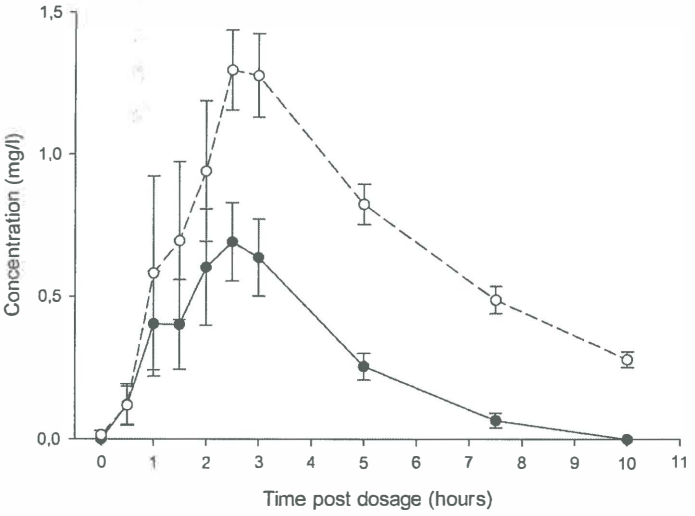

FIG. 2: CLA/14OH-CLA concentration-time curve. Mean (standard error) plasma concentration-time curves for CLA (solid circles) and 14OH-CLA (open circles) from the 4SR/CR4 arm are shown.

Table 2: Pharmacokinetics of rifampicin and clarithromycin in both treatment arms

Pharmacokinetic parameter	Result for each treatment arm[a]						*P* value for difference in RIF
	4SR/4CR (n = 8)				8SR (n = 5)		
	CLA	14OH-CLA	RIF	25DA-RIF[b]	RIF	25DA-RIF[c]	
AUC_{0-24h} (mg • h/liter)	2.9 (1.5 – 3.8)	8.6 (6.8 – 9.0)	25.8 (21.9 – 31.5)	4.2 (3.2 – 5.1)	16.2 (15.0 – 17.6)	1.1	0.09
C_{max} (mg/liter)	1.0 (0.5 – 1.3)	1.5 (1.2 – 2.1)	4.9 (3.3 – 6.4)	0.6 (0.4 – 0.6)	4.2 (4.0 – 4.3)	0.4	0.35
T_{max} (h)	2.3 (1.6 – 2.9)	2.9 (2.4 – 3.5)	3.6 (3.2 – 4.4)	4.1 (3.3 – 4.4)	3.5 (2.5 – 3.9)	0.6	0.62
$t_{1/2}$ (h)	1.3 (1.0 – 1.4)	2.6 (2.3 – 2.9)	2.1 (2.0 – 2.2)	2.2 (1.3 – 3.1)	2.3 (1.9 – 2.4)	0.1	0.35
CL/F (liter/h)	89.2 (68.8 – 151)	30.9 (19.7 – 41.3)	11.9 (8.8 – 15.2)	132 (109 – 156)	18.5 (17.0 – 21.1)	89.7	0.09
V_d/F (liter)	174 (144 – 214)	141 (117 – 230)	38.1 (26.4 – 50.8)	425 (243 – 632)	58.6 (57.4 – 59.8)	13.5	0.27

[a] The values are medians (IQRs).
[b] n = 4.
[c] n = 1.

Discussion

The present study investigated the interaction between RIF and CLA in patients infected with *M. ulcerans* as part of a prospective randomized trial comparing two drug regimens. We observed that the median total RIF exposure was increased 60% in patients receiving CLA compared to that in patients receiving RIF combined with SM. This may be explained by the inhibitory effect of CLA on the Pgp-mediated efflux of RIF and not by the inhibitory effect of CLA on CYP3A4, as RIF is not a CYP 3A4 substrate.

Our study had a relatively small sample size, and due to the high degree of variability in the results, only a trend toward significance could be shown. Although the difference was not significant in our study, it might still be relevant for clinical practice, as the increased exposure to RIF could have contributed to the efficacy of the drug treatment because the RIF AUC/MIC ratio is one of the best parameters predicting the efficacy of treatment of infections caused by *Mycobacterium tuberculosis* (23). No differences in the concentration of 25DA-RIF were seen between the treatment arms. Due to the low of level exposure of 25DA-RIF in plasma, no additional bactericidal effect can be expected. The only effect of this metabolite may be expected in human bile, in which it tends to accumulate (22). The effect of RIF on CLA is more difficult to explain, as the present study lacked a study arm without RIF. We observed that the level of exposure to 14OH-CLA was significantly higher than that to CLA. The median 14OH-CLA concentration-time curve (Fig. 2) is higher than the median CLA concentration-time curve for the time period observed. This indicates that the metabolism of CLA to 14OH-CLA is induced by RIF, as the 14OH-CLA concentration-time curve is higher than the CLA concentration-time curve. This is consistent with the findings of earlier studies (28, 37). The ratio of the parent drug to metabolite is, however, less than that reported earlier (28).

The results of our study are in line with the observations of the interaction of CLA and rifabutin (RBN), which has been studied in volunteers infected with HIV. Rifabutin, like rifampin, induces Pgp and the CYP 450 system, but less profoundly than RIF does (2). In the present study, the CLA-RBN interaction resulted in a significantly decreased AUC for CLA compared to that before the introduction of RBN, but the 14OH-CLA concentration increased significantly along with a significant increase in RBN drug concentrations (19).

Our results are consistent with earlier data that the ratio of the C_{max} of CLA to the C_{max} of 14OH-CLA is reduced to a greater extent when CLA is combined with RIF (28) than when RBN is combined with RIF (37) and no inducer is coadministered. The major concern in the case of these drug-drug interactions is that the resulting

antibiotic exposure is not efficacious due to the altered plasma concentrations. The decrease in CLA levels must therefore be compensated for by the increase in 14OH-CLA level and can be expressed as the ratio of the CLA level/14OH-CLA level. Whether the observed ratio is sufficient for efficacious treatment depends on the in vitro susceptibility of the targeted microorganism to both CLA and 14OH-CLA. In our study we observed that the concentration of CLA in plasma was above the MIC of 0.25 to 0.5 mg/liter for *M. ulcerans* for a median period of at least 120 to 248 min. As the MIC for 14OH-CLA was 4 to 8 mg/liter, it was presumed that only CLA contributed to the bactericidal activity of the agent, as the level of exposure to 14OH-CLA was always below the MIC. In other bacterial infections, CLA can be efficacious if the concentration is above the MIC for some part of the dosing interval (1). The time above the MIC for CLA therefore seems to be less important than it is for, e.g., beta-lactam antibiotics and is difficult to correlate with efficacy (1). In addition, the plasma concentration of CLA does not necessarily reflect its intracellular bactericidal efficacy, as efficacy depends on the distribution of the antibiotic into the tissue and cells (6). Although the CLA concentration in the interstitial fluid of the skin might be lower than that in plasma (35), the concentrations of CLA in cells are expected to be higher than those in plasma (32).

The present study was limited by the fact that neither the distribution of the antibiotic into the tissue and cells nor the concentration in the interstitial fluid of the skin was determined. On the basis of the results of the BURULICO trial, both CLA and RIF exposures appeared to be sufficient for the treatment of *M. ulcerans* infection, as no significant difference (one-sided Fisher's exact test, $P = 0.16$) in the healing rate was observed between the 8SR (96%) and 4SR/4CR (91%) arms (26). As 5 of the 75 subjects treated with CLA appeared to be positive for *M. ulcerans* as a result of the non-protocol-driven testing of samples by culture, there are still some doubts about the total antibiotic exposure. Although the coadministration of RIF resulted in a lower level of CLA exposure, it should be mentioned that CLA was given at a low dose of 7.5 mg/kg once daily. During the design of the randomized controlled trial, concerns about the safety of CLA at a dose of 7.5 mg/kg twice daily resulted in the use of this dosage scheme.

On the basis of these clinical results, it might be hypothesized that RIF was effective in reducing the *M. ulcerans* bacterial load or at least stopping mycolactone production, while CLA prevented the emergence of microorganisms resistant to RIF (25). The contribution of CLA is uncertain, however, and measuring the intracellular concentrations CLA would be the best method to assess the combined extra- and intracellular exposures of *M. ulcerans* to CLA. Animal (mouse) model studies

of *M. ulcerans* infection have evaluated only the correlates between dosages and the bacteriological response and clinical effects, especially in the footpad model (11, 12, 24). Future animal studies should address PK aspects, along with these clinical and bacteriological responses, to develop PK/pharmacodynamic models for the prediction of outcomes.

Table 3. Rifampin population pharmacokinetic model parameters values[a]

Parameter	Result (mean ± SD) for each treatment arm		*P* value
	4SR/4CR	8SR	
CL (liters/h/1.85 m^2)	24.1± 9.8	37.3 ± 5.5	0.17
Vd (liters/kg LBMc)	1.19 ± 0.21	1.43 ± 0.10	0.17
k_a (h^{-1})	0.998 ± 1.35	0.807 ± 0.574	0.83
T_{lag} (h)	2.07 ± 0.55	1.52 ± 0.86	0.44

[a] Oral bioavailability was fixed at a value of 1 for both treatment arms.
[b] LBMc, lean body mass; k_a, absorption rate constant; T_{lag}, lag time; the other abbreviations are defined in the text.

In further clinical studies, a regular CLA dose of 7.5 mg/kg twice daily or a CLA slow-release formulation at a dose of 15 mg/kg once daily should be used to ensure higher levels of exposure to CLA and increase the time above the MIC compared to those achieved with the currently used dose of 7.5 mg/kg once daily, which appeared to be safe and well tolerated.

Conclusion

Combination of RIF and CLA resulted in a considerable but nonsignificant increase in plasma RIF levels compared to the RIF levels achieved with coadministration of RIF and SM. A decrease in plasma CLA levels and an increase in plasma 14OH-CLA levels were observed, but the time that the concentrations of CLA in plasma were above MIC of *M. ulcerans* was reached for at least a part of the day.

Acknowledgments

The purified clarithromycin drug substance and metabolite were kindly provided by Abbott, and the purified rifampin drug substance and metabolite were kindly provided by Sanofi Aventis (Germany). Both *M. ulcerans* strains were generously provided by Pamela Small, University of Tennessee, Knoxville, TN.

W. A. Nienhuis and T. S. van der Werf were supported by grant EU FP6 2003-INCO-Dev2-015476. A. T. Zuur was supported by Stichting BuG. We have no conflicts of interest to declare.

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CHAPTER 3.2

Antimicrobial drugs for Buruli ulcer

Lancet. 2010 May 29;375(9729):1873; author reply 1873-4

In their Article on antimicrobial therapy for early, limited *Mycobacterium ulcerans* infection in Ghana (Feb 20, p 664),[1] Willemien Nienhuis and colleagues conclude that 4 weeks of streptomycin plus rifampicin followed by 4 weeks of rifampicin and clarithromycin has similar efficacy to 8 weeks of streptomycin and rifampicin. Some concerns about the use of clarithromycin in Buruli ulcer need discussing.

Treatment failure was higher in the 4-week streptomycin plus 4-week clarithromycin group than in the 8-week streptomycin group. Mycobacterium spp carry many macrolide resistance traits in their chromosomes and can confer high resistance to clarithromycin after exposure to similar classes of drug.[2] One study in a mouse model revealed that, when given at a daily dose of 100 mg/kg (equivalent to 1 g per day in human beings), clarithromycin had an obvious bacteriostatic activity: the growth of *M ulcerans* in mice treated with clarithromycin was significantly delayed compared with that in control mice.[3] However, since the activity was not bactericidal, one may question the potential practical value of clarithromycin in the treatment of *M ulcerans* infection in human beings. The high rate of treatment failure in the 4-week clarithromycin group in Nienhuis and colleagues' study could reflect this limitation.

Moreover, it is now generally accepted that a major protective antigen present in the Bacille Calmette-Guerin (BCG) vaccine is the so-called Ag85 complex which has 84% sequence identity and 91% similarity with *M ulcerans* Ag85A proteins.[4] Logically, then, BCG-vaccinated patients should recover more quickly from Buruli ulcer than non-vaccinated individuals.

I have been supported by the Canada Institute of Health Research. I declare that I have no conflicts of interest.

Dewan S Billal

Centre de Recherche en Infectiologie, RC709 CHUQ, Québec, QC G1V 4G2, Canada

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We appreciate and to a large extent share the concern of Dewan Billal about the role of clarithromycin in the management of Buruli ulcer disease. Although patients who switched to oral treatment with rifampicin and clarithromycin had a statistically similar beneficial response to treatment, assessment of the added effect (if any) of clarithromycin is impossible. Indeed, presently the duration of therapy necessary to switch off mycolactone production and allow the immune system to recover and clear residual bacteria is unclear. Basically, we added clarithromycin to include an oral drug that was tested in vitro[1] to prevent monotherapy with rifampicin and to limit the number of streptomycin injections. During the trial, in-vivo data were published that supported this choice.[2]

We used a dose of 7·5 mg per kg bodyweight, once daily, as approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Our concern was that rifampicin co-medication might lower clarithromycin plasma concentrations by inducing cytochrome P450 isoenzymes. Preliminary data from a substudy suggest that clarithromycin drug concentrations are lower than the 14-hydroxy metabolite of clarithromycin.[3] Further more, preliminary results suggest that this metabolite does not con tribute to the antimicrobial activity against *M ulcerans*. Yet the median concentration of clarithromycin was above the minimal inhibitory concentration (MIC)—between 0·5 and 2·0 mg/L for most *M ulcerans* strains tested.[4] One should also consider accumulation of macrolide drugs in immune cells that might add to the clinical response seen, but the relatively short time above MIC for extracellular microorganisms is a concern if we are to consider testing a fully oral treatment schedule.

Although we did not examine BCG scars in our study participants, most children in Ghana receive BCG vaccination in the first week after birth; the protection by BCG vaccination is short-lived and rather weak.[5] BCG vaccination status is therefore unlikely to present a bias for the beneficial effect of antimicrobial treatment in both study groups.

Eventually, a fully oral treatment would be highly desirable. Before a trial is undertaken, an extended-release formulation of clarithromycin combined with rifampicin, approved by the FDA and EMA, should first be tested. With this information, we can select the best possible rifampicin-based combination regimen for oral treatment, and compare it with the present WHO-standard regimen of 8 weeks of streptomycin injections plus rifampicin.

We declare that we have no conflicts of interest.

Tjip van der Werf, Willemien Nienhuis, Jan Willem Alffenaar, Deepak Almeida, Ymkje Stienstra

University Medical Centre Groningen, University of Groningen, PO Box 30 001, 9700 RB Groningen, Netherlands (TvdW, WN, JWA, YS); and Johns Hopkins University, Baltimore, MD, USA (DA)

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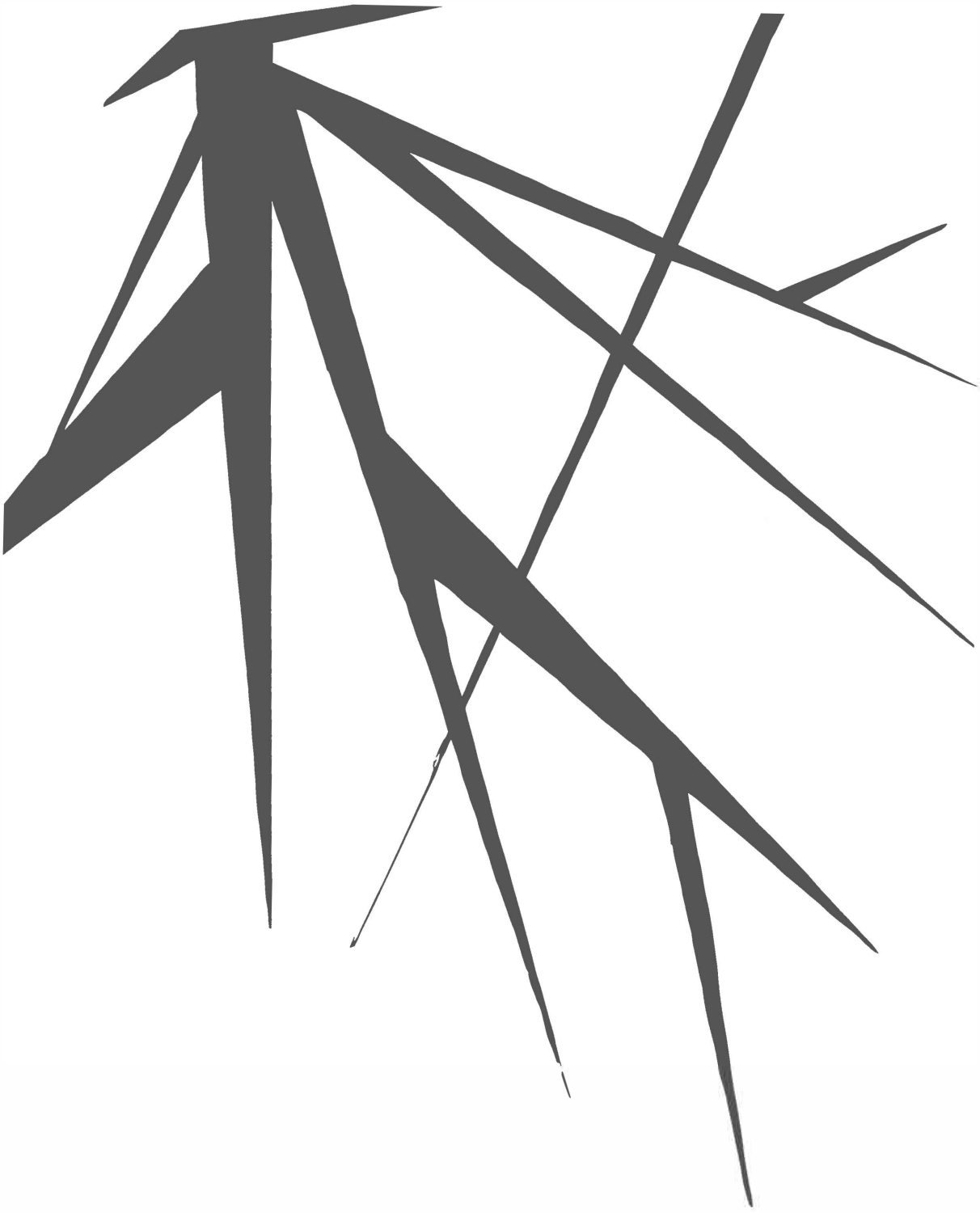

CHAPTER 4

Paradoxical responses after start of antimicrobial treatment in Mycobacterium ulcerans infection

Willemien A. Nienhuis
Ymkje Stienstra
K. Mohammed Abass
Wilson Tuah
William A. Thompson
Peter C. Awuah
Nana Yaa Awuah-Boateng
Ohene Adjei
Gisela Bretzel
Jan P. Schouten
Tjip S. van der Werf

Clin Infect Dis. 2012 Feb;54(4):519-26

Summary

Background Antimicrobial killing in mycobacterial infections may be accompanied by (transient) clinical deterioration, known as paradoxical reaction. To search for patterns reflecting such reactions in the treatment of Buruli ulcer (*Mycobacterium ulcerans* infection), the evolution of lesions of patients treated with antimicrobials was prospectively assessed.

Methods The lesion size of participants of the BURULICO antimicrobial trial (with lesions ≤10 cm cross-sectional diameter) was assessed by careful palpation and recorded by serial acetate sheet tracings. Patients were treated with antimicrobials for 8 weeks. For the size analysis, participants whose treatment had failed, had skin grafting, or were co-infected with human immunodeficiency virus were excluded. For every time point, surface area was compared with the previous assessment. A generalized additive mixed model was used to study lesion evolution. Nonulcerative lesions were studied using digital images recording possible subsequent ulceration.

Results Of 151 participants, 134 were included in the lesion size analysis. Peak paradoxical response occurred at week 8; over 30% of participants showed an increase in lesion size as compared with the previous (week 6) assessment. Seventy-five of 90 (83%) of nonulcerative lesions ulcerated after start of treatment. Nine participants developed new lesions during or after treatment. All lesions subsequently healed.

Conclusions After start of antimicrobial treatment for Buruli ulcer, new or progressive ulceration is common before healing sets in. This paradoxical response, most prominent at the end of the 8-week antimicrobial treatment, should not be misinterpreted as failure to respond to treatment.

Clinical Trials Registration ClinicalTrials.gov, NCT00321178.

Introduction

Buruli ulcer (BU), caused by *Mycobacterium ulcerans*, is a devastating mycobacterial disease [1–3]. It is the third most common mycobacterial infection in immunocompetent humans after tuberculosis and leprosy, and BU even outnumbers these infections in some areas in West Africa. Although most commonly seen in children, all age groups can be affected. It starts as a nodule, papule, plaque, or edema. If progressive, the skin breaks down and a typical painless ulcer appears with a necrotic center and undermined edges, surrounded by an indurated area. Surprisingly few patients present with accompanying systemic responses such as fever and malaise, even though ulcers may cover a substantial part of the body. Treatment, until recently, has been wide surgical excision of affected skin and surrounding normal tissue. In the past, antibiotics have been largely abandoned on the basis of disappointing field observations [4]. In a pilot study with antimicrobial end points, combination therapy with streptomycin and rifampicin was effective, and subsequent case series showed a beneficial clinical response [5, 6]. In the BURULICO trial we showed that early, limited BU lesions heal with antimicrobial treatment alone, without surgical debridement, in .90% of cases [7]. An additional 3 reports have supported the beneficial effects of antimicrobial therapy, 2 of them including larger lesions as well [8–10].

In tuberculosis and leprosy, effective antimicrobial killing may be accompanied by (transient) clinical deterioration [11–13]. In these diseases the deterioration is known as a paradoxical or a reversal (type 1) reaction. A paradoxical reaction in tuberculosis is defined as a transient worsening of a preexisting lesion or the development of new lesions under appropriate therapy—some definitions incorporating initial improvement—affecting 2.4%–10% of individuals at time point 8–10 weeks (median) after start of treatment [12, 14, 15]. The incidence of paradoxical reactions, which has also been described in intracerebral tuberculomas [12, 16], is probably highest in lymph node tuberculosis [11, 17]. These reactions have been attributed to increased exposure to mycobacterial antigens, a decrease in suppressor mechanisms, or improved host cell–mediated immunity following bactericidal therapy [11, 15].

In the BURULICO trial [7], participants were followed up by analyzing serial surface area measurements of lesions and by digital imaging. Here we describe and compute responses compatible with a paradoxical reaction.

Methods

Study subjects

In the present analysis we included a subset of participants of the BURULICO drug trial, a randomized controlled trial for the treatment of early (duration <6 months), limited (cross-sectional diameter ≤10 cm) *M. ulcerans* infection. Polymerase chain reaction (PCR) was the planned confirmation test for *M. ulcerans* infection, which was achieved in 95% of all participants. We compared 8 weeks of treatment with streptomycin and rifampicin, with 4 weeks of treatment with streptomycin and rifampicin followed by 4 weeks of rifampicin and clarithromycin.

The primary outcomes of this trial have been reported elsewhere [7]. To study the lesion evolution during effective antimicrobial treatment, defined as healed by week 52, participants whose treatment had failed, had skin grafting, or were coinfected with human immunodeficiency virus (HIV) were excluded. Treatment failure was recorded if a lesion had not healed by 52 weeks, lesion recurred within 1 year, or lesion size increased to 150% at any time point compared with baseline, with surgical debridement undertaken as deemed necessary by the attending doctor. The trial was conducted at 2 different hospital sites in the Ashanti region of Ghana.

Study design

To determine the pattern of healing in response to antimicrobial treatment, lesions were examined by inspection followed by careful palpation. The lesion circumference, as assessed by the above methods, was recorded on serial acetate sheet tracings, and digital images were made of each participant's lesion. Participants were treated for 8 weeks and followed up for 1 year after start of treatment. Acetate sheet tracings and digital images were made at start of treatment, and at week 2, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32, 36, 44, and 52, until complete healing (defined as complete reepithelialization) occurred. For participants with more than 1 lesion, only the largest was measured.

Methods of measurements

The members of the BU study teams that drew the lesions were instructed to palpate the extent of the indurated area around the visible skin defect and to include this induration in the tracing. Before treatment was started, from every non-ulcerated lesion 3 3-mm punch biopsies were taken under local anesthesia for diagnostic confirmation, including culture, microscopy, PCR [18], and histopathology. From ulcerated lesions, in addition to punch biopsies, diagnostic swabs were taken [7]. The acetate sheet recordings were disinfected and stored in the patient record files. At

the end of the study all sheets were scanned, and the surface area was calculated for every individual lesion at the different time points, using Adobe Photoshop (version CS3 Extended). Digital camera images were uploaded and stored in anonymized patient folders.

Analysis of data

Results of participants of the 2 antimicrobial regimens were taken together, as no significant differences were found for overall healing (healing at 1 year after start of treatment without recurrence or large debridement surgery) and time to healing. To precisely evaluate the effect of treatment, ongoing healing was recorded by comparing every lesion measurement to the preceding assessment and arranging them in 1 of 3 groups: (1) lesion size increased compared with last assessment, (2) size decreased compared with last assessment, and (3) lesion healed.

Results were plotted over time. Generalized additive mixed models (GAMM) analysis [19] was used to create best-fitting curves for mean rate of healing over time, with allowance for group-specific and subject-specific smooth deviations and missing values. GAMM is a fixed-effects regression model with random-effect terms in addition to the fixed effects and with unknown smooth functions of some of the covariates making it appropriate for analyzing clustered—and therefore dependent—data sampled over time, with missing values and with a nonlinear course (smooth) of the dependent variable with time. As basis functions, the truncated powers basis with 14 knots was used, with 90% confidence intervals (CIs) based on a bias-adjusted approximation of the covariance matrix.

Lesions that were not measured because they were larger than the sheets used for drawing were analyzed as "previous size measurement +1." Type of treatment combination, ulceration, and lesion category at inclusion were explored as possible influencing factors on lesion evolution. Category I comprises lesions <5 cm, category II lesions between 5 and 10 cm, and category III lesions >10 cm, multiple lesions, or lesions at critical sites. Several definitions of a paradoxical increase in surface area were depicted and percentages of participants were calculated meeting these criteria for every time point and for the overall study period. The percentage of participants with non-ulcerative lesions that subsequently ulcerated was calculated. Data were analyzed and figures drawn using SPSS software (version 16.0, IBM SPSS) and R software (R version 2.13.0, R Foundation for Statistical Computing) and Package Amer (additive-mixed models with lme4, R package version 0.6.10).

Ethics

The Medical Ethics Review Committee of the University Medical Centre Groningen, the Netherlands, reviewed the protocol of the trial. The protocol was approved by the Committee on Human Research, Publication, and Ethics of the Kwame Nkrumah University of Science and Technology and the Komfo Anokye Teaching Hospital, Kumasi (CHRPE/07/01/05), and by the Ethical Review Committee of Ghana Health Services (GHS-ERC-01/01/06). Written and verbal informed consent or assent was obtained from all participants aged ≥12 years, and consent from parents, caretakers, or legal representatives of participants aged ≤18 years.

Table 1: Baseline characteristics of the participants included in the *Mycobacterium ulcerans* lesion size analysis (n=134)

	No. (%) (*n* = 134)
Sex, male	41 (31)
Age, years, median (IQR)	12 (8-20)
Study site	
Agogo	95 (71)
Nkawie	39 (29)
Lesion surface area, cm^2, median (IQR)	25 (9-45)
Category of lesion at inclusion	
I	55 (41)
II	77 (57)
III	2 (2)
Type of lesion at inclusion	
No ulceration	85 (63)
Ulceration	49 (37)
Randomization group	
8 weeks streptomycin/rifampin	69 (51)
4 weeks streptomycin/rifampin and 4 weeks clarithromycin/rifampin	65 (49)

Abbreviation: IQR, interquartile range.

Results

Patient population

Table 1 shows baseline characteristics of the participants. Of the 151 participants included in the BURULICO antimicrobial trial, 17 were excluded: 10 who had experienced treatment failure; 3 who were coinfected with HIV (of whom 1 experienced treatment failure); and 5 who had received skin grafts. Therefore, 134 participants were included for the acetate sheet measurements analysis. Of those, 129 (96%) were confirmed BU patients (124 by PCR; 3 by culture; 1 by histopathology; 1

by microscopy). Five (4%) were not confirmed. Sixty-three of 134 participants (47%) missed at least 1 of the 15 scheduled assessments. One participant's lesion was not measured at 1 time point because of the extent and was included as described in the Methods.

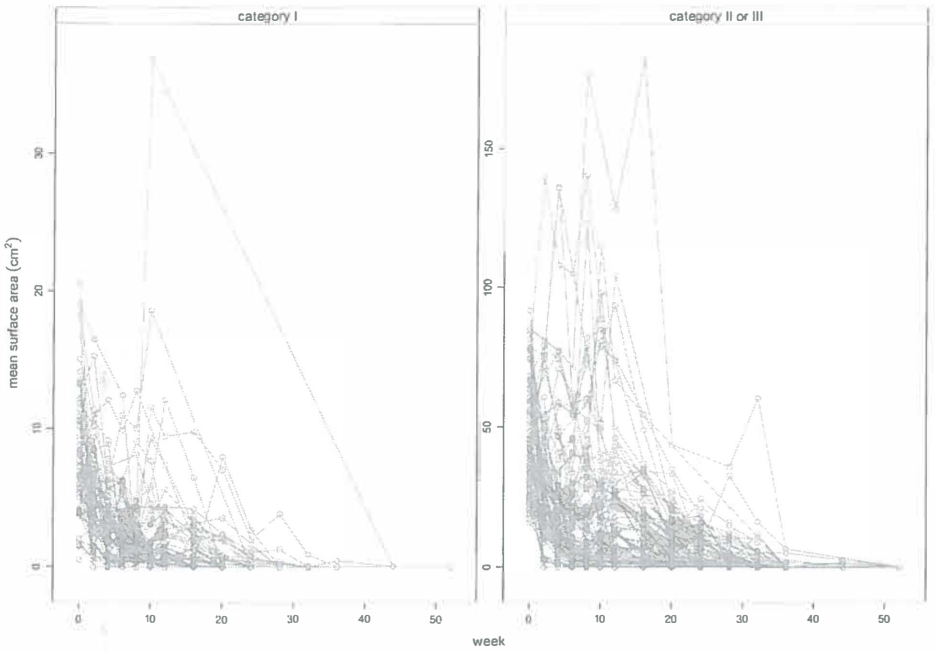

Figure 1: Course in surface area measurements of *Mycobacterium ulcerans* lesions plotted for every individual during and after 8 weeks of antimicrobial therapy. The number of *M. ulcerans* participants analyzed is 134 (all healed trial participants except skin-grafted and human immunodeficiency virus–coinfected persons). Category I (n = 55) is lesions < than 5 cm, category II (n = 77) is lesions between 5 and 10 cm, and category III (n = 2) is lesions > 10 cm, multiple lesions, or lesions at critical sites.

Evolution of BU lesions during and after treatment

Figure 1 shows the changes in lesion surface area plotted for every individual participant per lesion category. Figure 2 shows the percentage of participants in the 3 different groups (increased, decreased, or healed) for every time point, including the number of missing assessments. After 2 weeks of treatment, approximately 18% of the lesions showed an increase in size compared with the previous assessment. This percentage increased by approximately 30% at weeks 8, 10, and 12. The highest percentage of individuals with increased lesion size was recorded at week 8, with

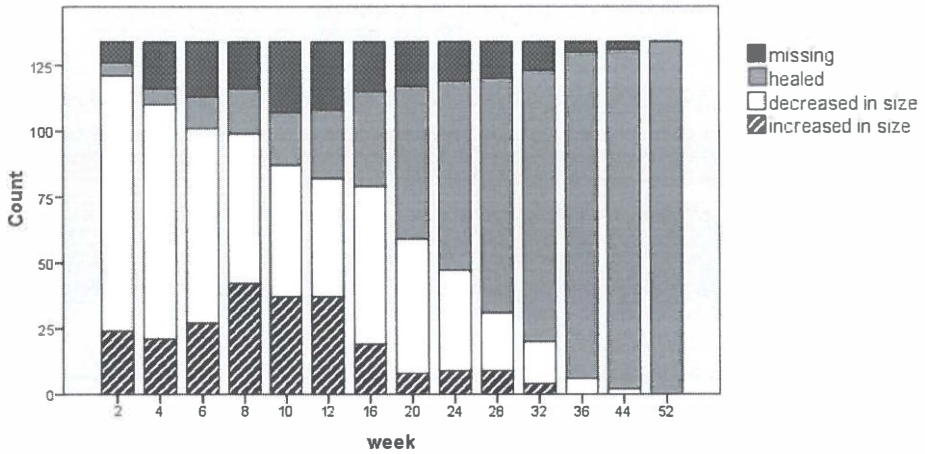

Figure 2: Patterns of response of *M. ulcerans* lesions during and after 8 weeks of antimicrobial therapy. Total count of lesions that showed complete healing (gray), an increase (hatched) or a decrease (white) in size as compared with the previous assessment, and missing assessments (black) are shown. The number of *M. ulcerans* participants analyzed is 134 (all healed trial participants except skin-grafted and human immunodeficiency virus–coinfected persons).

Overall, 105 of 134 participants (78%) showed an increase in lesion size compared with the preceding assessment, at some point in time. Increase in lesion size in excess of size at start of treatment was recorded in 40 of 134 participants (30%). Increase in lesion size in excess of 150% was recorded in 19 of 134 participants (14%). According to the protocol, these 19 participants were discussed with the attending medical staff for possible debridement surgery, but as the overall clinical assessment was not unfavorable, with decrease in wound depth, edema, and necrosis, they were not operated on. Overall, healing was steady but slow.

Table 2 shows the percentages of participants who met various exploring criteria that defined paradoxical responses in the pattern of healing. For example, when using an increase in surface area after a decrease in the preceding measurement, approximately 16% of participants met the definition at week 10 (measured at weeks 8 and 10) and week 12 (measured at weeks 10 and 12). Using the stricter criterion of 2 consecutive increases after 1 decrease, 7.5% of participants met the definition at week 10 (measured at weeks 6, 8, and 10).

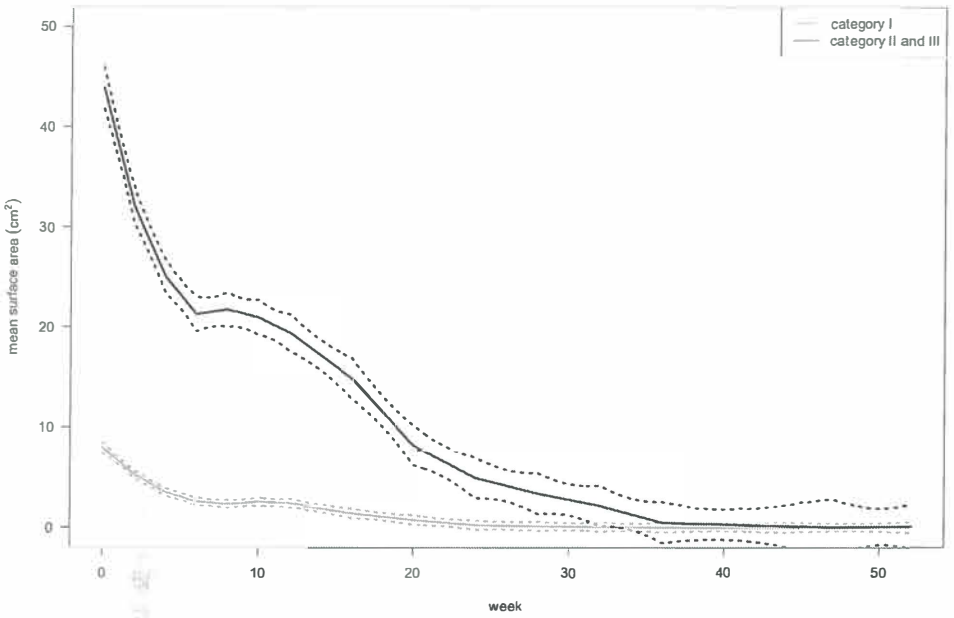

Figure 3: Generalized additive mixed model analysis for mean lesion surface area, for lesion category subgroups (with smooth subject-specific deviations from group specific curves; see text for details). The dotted lines show the upper and lower 90% confidence intervals. The number of *M. ulcerans* participants analyzed is 134 (all healed trial participants except skin-grafted and human immunodeficiency virus–coinfected persons). The number of category I *M. ulcerans* lesions was 55; the number of category II and III lesions was 79.

Non-ulcerative lesions with subsequent ulceration before healing and new lesions

Ninety-two of the 151 participants in the BURULICO anti-microbial trial had non-ulcerative lesions. From these, 2 had treatment failure and 2 were HIV co-infected. Of the remaining 88 participants, 15 (17%) never had ulceration; they subsequently healed. The other 73 (83%) developed ulcers after a median period of 6 (interquartile range, 2–8) weeks but nonetheless healed subsequently (Table 3). Sixteen of these 73 participants developed the ulcer after completion of antimicrobial treatment. Some participants reported increase of pain at the lesion site just before and at the time the lesion ulcerated.

Disease-confirming punch biopsies were taken from at least 1 lesion of all included participants before start of treatment. Yet some participants had >1 lesion (only the largest lesion had a biopsy taken) or developed new lesions during or after treatment. Eleven participants had a total of 16 non-ulcerative lesions that were not biopsied. Ulceration likewise occurred in 15 of 16 lesions that did not have earlier punch

biopsies; all of these lesions healed after the ulceration. One lesion was excised in a non-ulcerative state. Thirteen of 151 participants (9%) had >1 lesion at any point in time. Four participants had >1 lesion at start of treatment. In 5 participants, the second lesion became apparent during antimicrobial treatment. From 4 of these participants, swabs were taken at time of ulceration; 2 were microbiologically confirmed to be *M. ulcerans* lesions—1 by PCR and 1 by microscopy. In 4 participants the second lesion appeared after treatment completion; 1 of these also developed a third lesion. From 4 of these 5 lesions, swabs were taken when the lesion ulcerated; 2 were confirmed by PCR and 1 by culture. All of these lesions, except for 1 that was excised, ulcerated spontaneously and healed subsequently without further intervention.

Table 2: Several definitions for paradoxical evolutions in surface area in ultimately healed participants with Mycobacterium ulcerans lesions per time point.

Week	1 increase in SA, No. (%)	1 increase in SA after 1 decrease, No. (%)	2 increases in SA, No. (%)	1 increase in SA after 2 decreases, No. (%)	2 increases in SA after 1 decrease, No. (%)
2	24 (17.9)	...	...	...	...
4	21 (15.7)	14 (10.4)	7 (5.2)	...	...
6	27 (20.1)	22 (16.4)	3 (2.2)	18 (13.4)	2 (1.5)
8	42 (31.3)	31 (23.1)	8 (6.0)	27 (20.2)	6 (4.5)
10	37 (27.6)	22 (16.4)	13 (9.7)	15 (11.2)	10 (7.5)
12	37 (27.6)	21 (15.7)	15 (11.2)	10 (7.5)	9 (6.7)
16	19 (14.2)	10 (7.5)	5 (3.7)	6 (4.5)	3 (2.2)
20	8 (6.0)	7 (5.2)	1 (0.7)	2 (1.5)	0 (0)
24	9 (6.7)	7 (5.2)	2 (1.5)	6 (4.5)	2 (1.5)
28	9 (6.7)	9 (6.7)	0 (0)	7 (5.2)	0 (0%)
32	4 (3.0)	3 (2.2)	0 (0)	2 (1.5)	0 (0%)
Overall[a]	105 (78.4)	94 (70.1)	39 (29.1)	76 (56.7)	31 (23.1)

Criteria incorporate consecutive measurements. Percentages are taken from all healed trial participants except skin-grafted and human immunodeficiency virus–coinfected persons (n = 134). Missing measurements varied from 0.7% to 20.1% per time point.
Abbreviation: SA, surface area.
[a] Number of participants meeting the criterion at least once during the 52 weeks.

Discussion

We describe a response pattern following antimicrobial therapy for BU that we characterize as paradoxical reaction. This paradoxical reaction appears to be common and unexceptional. We show that during and shortly after anti-mycobacterial treatment for BU, 23% (using 2 consecutive measurements with an increase after 1 measurement with a decrease) to 78% (using 1 measurement) of lesions increased in size, and 83% of non-ulcerated lesions progressed to ulcers. Six percent of the participants enrolled developed lesions that were not clinically present at start of treatment. Eventually, all of these lesions healed within a follow-up period of 1 year. The number of lesions that increased in size compared with the previous assessment peaked at weeks 8, 10, and 12—just at the end of the 8-week treatment period. Paradoxical reactions in tuberculosis seem to appear around the same time [14, 15]. Our results indicate that in BU, paradoxical reactions may be more common. It might also be that the frequency of paradoxical reactions is comparable but more apparent in BU, because it affects the skin. Two studies that specifically looked at lymph node tuberculosis, in which paradoxical reactions are more objectively measurable than in pulmonary tuberculosis, reported transient enlargements in approximately 25% [11, 17], comparing more favorably with our results for BU. A recent publication on the efficacy of streptomycin combined with rifampicin in a study in Ghana also describes paradoxical responses, but not in the same frequencies as we describe [8].

Earlier observations in BU that lesions progress during antimicrobial treatment may have been misinterpreted as lack of antimicrobial killing. Two reports from Australia have suggested that paradoxical reactions in BU occur [20, 21]. In a trial in Uganda, the study drug, clofazimine, was probably not active; therefore, possible paradoxical reactions cannot be evaluated [22]. The paper, however, provides unique information on placebo-treated individuals—and those on clofazimine—that healed by virtue of their protective immune response without active drugs or surgery. Although participants had no disease confirmation, the spontaneous healing rate was 11 of 34 participants (32%). In participants with non-ulcerated lesions who received placebo, 10 of 11 participants progressed to ulcers before spontaneous healing occurred. In a drug trial for BU in which 2 months of rifampicin and dapsone were compared with placebo, lesion size was measured at 4 and 8 weeks after start of treatment [23]. Our results suggest that these time points may not be optimal to assess clinical success.

Table 3: Ulceration of non-ulcerative Mycobacterium ulcerans lesions after start of treatment in ultimately healed participants.

Week	New ulceration, No (%)
2	24 (27.3)
4	11 (12.5)
6	10 (11.4)
8	12 (13.6)
10	7 (8.0)
12	7 (8.0)
16	2 (2.3)
No ulceration	15 (17)

Of 88 healed, non-human immunodeficiency virus–infected participants with a nonulcerative lesion at start of treatment, ulceration occurred in 73 (83%) at a median time of 6 weeks after start of treatment (25th-75th percentiles: 2-8 weeks).

We hypothesize that the transient clinical deterioration of lesions seen in our study is a result of restoration of local and systemic immune responses [24–27]. The lesions that appeared and healed spontaneously during and after treatment probably reflect an inflammatory response as well; the live microbes—or microbial antigens of dead bacilli—already present in tissue initially failed to elicit a host immune response. Mycolactone, the exotoxin produced and secreted by *M. ulcerans*, has been proposed as the major cause of immune suppression [24, 26–29]. The immunosuppressive signature appears to wane over time following effective antimicrobial treatment [30, 31], and we hypothesize that the pro-inflammatory response we describe as paradoxical response coincides with the wash-out of mycolactone from the lesion.

This study is limited by the fact that 47% of participants missed at least 1 of the 15 scheduled assessments. The highest numbers of missing measurements occurred at weeks 10 and 12, the time points that showed the highest increases in surface area. This means that the number of participants with paradoxical patterns might be larger than described. By using a GAMM, we were able to include participants with missing data in the analysis.

Another limitation is that measuring surface area of Buruli ulcers, which have an undermined edge and often a large indurated area, is difficult and not validated. A certain percentage of measurements will show an increase in surface area at the subsequent drawing, only by chance. However, stricter definitions (eg, ≥2 consecutive measurements of increase in lesion size after an initial decrease) confirm paradoxical evolutions after start of treatment.

We considered the possibility that the punch biopsies for disease confirmation may have elicited ulceration. We conclude from our study that ulceration is a common stage in the pattern of healing. Other lesions that were present at start of treatment

or appeared during or after treatment, and that were not biopsied, ulcerated as well. We assume that the ulcerative stage reflects the natural course of disease and is not necessarily related to the biopsy procedure.

Isolating live *M. ulcerans* from such an ulcerating nodule does not necessarily imply failure of treatment. In the original report of the BURULICO trial [7], we described a patient who developed a nodule 8 weeks after treatment completion. This nodule ulcerated 6 weeks later, and *M. ulcerans* was cultured from this lesion. This lesion healed without further intervention.

In conclusion, we describe new and progressive ulceration and appearance of new lesions in *M. ulcerans* infection, reflecting paradoxical reactions following antimicrobial treatment. These reactions were common and occurred during and after antimicrobial treatment. We show evidence from the literature that they mirror an enhanced host immune response. The time course might be compatible with arrest in production and gradual decrease of mycolactone concentrations in lesions, and possibly systemically, with restoration of the local and systemic host immune responses against mycobacterial antigens that were first suppressed by mycolactone. The evolution of lesions after start of antimicrobial treatment could be detected by careful observation, avoiding early referral for surgery. Further studies are required to improve our understanding of mycolactone kinetics over time and to link such data with host response patterns. Paradoxical reactions in BU may earlier have gone unrecognized and been misinterpreted as failure to respond to treatment. A precise definition of paradoxical response would be welcomed, before a clinical decision rule can be developed to differentiate this entity from treatment failure. This information is important in the design of future clinical studies as well as in the management of patients with BU disease [32].

Author contributions

T. S. W., W. A. N., G. B., and Y. S. designed the study. K. M. A., W. T., and W. A. N. performed the acetate sheet measurements. W. A. T. and P. C. A. advised on clinical judgment and decisions. J. P. S., W. A. N., and Y. S. performed the statistical analysis. W. A. N., Y. S., and T. S. W. wrote the paper. All authors helped in valuable literature appraisal and provided input in consecutive versions of the manuscript. All authors saw and approved the final version.

Acknowledgments

We thank all participants of the BURULICO drug trial and their parents, brothers and sisters, and other caretakers. We thank the BU team members at the 2 study

sites; the students who helped the BU teams; Richard O. Phillips for practical and expert advice; Auke-Willem Kampen for guiding the digital part of the work; and Harry J. Nienhuis for scanning the acetate sheets and for participation in calculating surface areas.

Financial support

This work was supported by the European Union (grants EU FP6 Burulico 0150476 and EU FP7 BuruliVac 241500), the Netherlands Organisation for Scientific Research (VENI grant to Y. S.), the Buruli ulcer Groningen Foundation, and Stichting Fonds Dr Catharine van Tussenbroek.

Potential conflicts of interest

All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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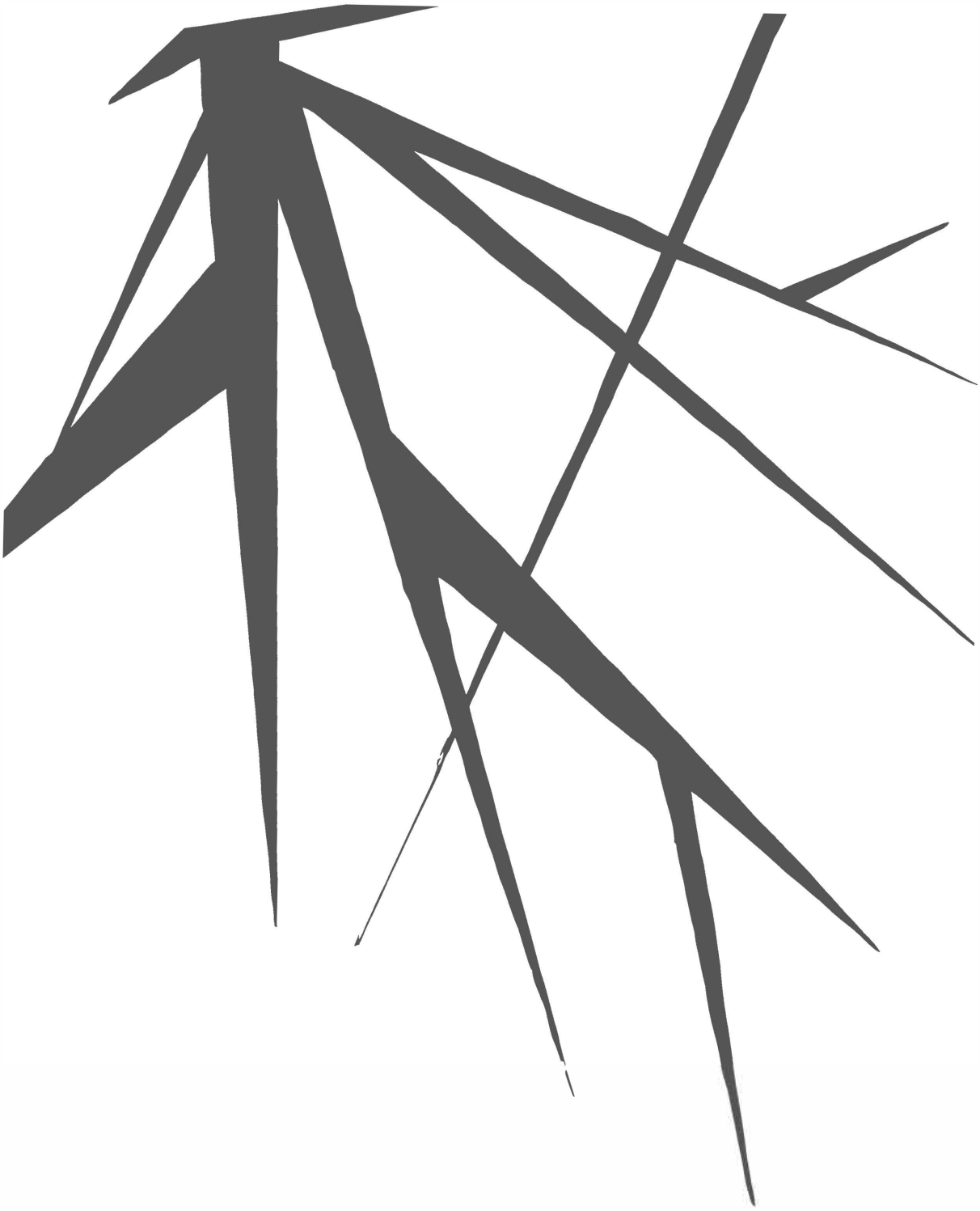

CHAPTER 5

Validity and test-retest reliability of portable pure tone audiometry in rural Ghana

Willemien A Nienhuis
Meys M Cohen
Ymkje Stienstra
Geoffrey K Amedofu
K Mohammed Abass
Wilson Tuah
Edmund Atakora
Grace Ocansey
Pieter U Dijkstra
Hero P Wit
Tjip S van der Werf

Manuscript submitted

Summary

Background Rural populations in developing countries have limited access to audiology services. In the context of a trial with a potentially ototoxic drug (the aminoglycoside streptomycin), we assessed the validity and test-retest reliability of audiometric monitoring with battery-powered portable equipment.

Methods For validity testing, readings of a portable conventional pure-tone audiometer (AS208; Interacoustics, DK-5610 Assens, Denmark) were compared with readings of fixed, standard equipment, both in a sound-proof setting. In a field study the intra- and inter-observer reliability were tested.

Results For comparison of standard and portable equipment, at least 96% of the repeated measurements in the 0.5 to 4 kHz range were within 10 dB. For intra- and inter-observer comparisons, at least 94% and 96% of the repeated measurements in the 0.25 to 4 kHz range were within 10 dB. At 6 and 8 kHz, repeatability was less. Mean ambient noise was high [50 dB(A)].

Conclusions To pick up changes in hearing (>10 dB) over time as a result of interventions, audiometric check-ups in the field with portable equipment are feasible, and reliable in the 0.25 to 4 kHz range. Absolute deviations from reference values should be interpreted with care, taking into account ambient noise. As aminoglycoside-induced ototoxicity (AIO) affects frequencies higher than 4 kHz first, early detection of AIO with portable audiometry in the field is limited.

Introduction

Hearing impairment is a problem worldwide. The WHO global estimate for disabling hearing impairment using a deficit >40 dB in the better ear in adults and >30 dB in children, has more than doubled between 1995 and 2005, from 120 million people to at least 278 million people (reference note 1). Of all deaf and hearing-impaired people, 80% live in low- and middle-income countries, and the number of children with hearing impairment is increasing [1].

Among the many causes of hearing impairment, acquired causes are more common in the less affluent rural communities [2,3]. Hearing impairment may be related to infections and treatment of infectious conditions. Aminoglycoside antimicrobial treatment has been an important cause of ototoxicity, especially among elderly patients with treatment of longer duration [4-10]. Some individuals are extremely vulnerable to aminoglycoside-induced ototoxicity (AIO) and have idiosyncratic toxicity due to mutations in the 12S subunit of ribosomal mitochondrial RNA [11]. Several different mutations in this mitochondrial gene are associated with AIO, the most important being the A1555G and 961 mutations [7,9,12]. Up to 33% of patients with AIO may have such mutations. Information from African populations, where aminoglycoside use is more widespread than in any other continent, is left with a description of AIO within a South African family, associated with the A1555G point mutation [13]. In Ghana, drugs were involved in only <2% of the causes of hearing impairment [2]. Because genetic testing identifies only individuals with idiosyncratic toxicity, and because pre-treatment genetic testing is not available, phenotypic testing must be used to demonstrate ototoxic effects [14]. Audiometry has been recommended for this purpose [5,9].

For most inhabitants of rural areas in developing countries, facilities to test hearing are not accessible. There are only a few otorhinolaryngology and audiology centers with soundproof audiometry facilities. The challenge is to provide audiometry services to reliably identify and refer subjects with impaired hearing for further investigation, treatment and/or counseling. Pure-tone audiometry using portable equipment has been used in less affluent areas in Africa [15,16]. Although pure tone audiometry has been tested for reliability in standard conditions with low ambient noise [17-19], it has not been tested for reliability under field conditions. Pure tone audiometry is basically subjective, but it appears to be reliable in cooperative subjects under controlled conditions [20]. The objectives of this study were (i) to determine the validity of pure-tone portable audiometry equipment by comparing readings in sound-proof conditions with standard equipment at the ENT department, Komfo-Anokye Teaching Hospital, Ghana; and (ii)

to determine intra- and inter-observer reliability of pure-tone portable audiometry in the 0.25 to 8 kHz frequency range in a rural hospital setting. In addition, we specifically related our results to ototoxicity screening, as pursuing reliable screening for possible AIO in the field was the initial reason for designing this study.

Methods

Study participants

Study participants for validity testing of the portable equipment were recruited among out-patients attending the ENT Department (head: GKA) and the tuberculosis unit of the Komfo-Anokye Teaching Hospital (KATH) in Kumasi. Participants in intra- and inter-observer reliability tests were recruited at two rural hospitals in the Ashanti Region, Ghana –Nkawie-Toase Governmental Hospital and Agogo Presbyterian Hospital. Most of these study participants were Buruli ulcer patients who were also included in the BURULICO drug trial comparing 4 or 8 weeks of the aminoglycoside antibiotic streptomycin as part of different drug combinations (trial registration number NCT00321178) [21]. For this drug trial the participants had to undergo audiometry regularly, and these assessments were integrated in the present study. Most of the drug trial study participants were between 5 and 15 years of age. Older participants were recruited from the ward, most of them admitted because of tuberculosis treatment. Participants were enrolled prospectively, in a consecutive order, during times the study team was present. Recruitment was done between August, 2006, and November, 2006.

For all tests, persons with a deficit of >90 dB hearing level (HL) for the worse ear and children under 5 years were excluded. Persons who were uncooperative or had difficulty in communicating were also excluded. Most of the volunteers did not speak English but rather Twii; English-Twii interpreters assisted by giving instructions during the instruction period and whenever necessary.

Validity testing, standard versus portable equipment, KATH

The equipment at the ENT department of Komfo-Anokye Teaching Hospital was chosen as reference standard, being the only available audiometry facility in the region. In the sound-proof room of the ENT department, one observer (GO: a trained audiology assistant) tested each individual study participant twice, using both fixed standard equipment and the portable equipment, in random sequence.

Intra- and inter-observer reliability, field studies

For intra-observer reliability testing, participants were tested twice by the same observer (MMC: a Dutch medical student). For inter-observer reliability three different observers (MMC, WT and EA) performed the measurements by forming three different observer-pairs. Each participant was tested twice, one time by each of the observers of one observer-pair. The second observer was blinded to the results of the first observer. The sequence of the observers within the pairs was alternated to prevent sequence effects.

The test room at Nkawie-Toase Governmental Hospital was an office in the public health department. In Agogo Presbyterian Hospital two test rooms were used. One was an isolation ward and the other was a room next to the consulting rooms near the outpatient department.

Instrumentation

In the audiometry unit in the Komfo-Anokye Teaching Hospital standard, fixed equipment was used (Kamplex AD27 Calibrated to ANSI (S3.6 - 1969) standard) for comparison with the portable equipment. The portable equipment consisted of full-range air conduction diagnostic audiometers, a model Interacoustics AS 208 Type 4 tone audiometer (for details and serial numbers see reference note 2 and 3) and matched circum-aural earphones with noise reducing Peltor mute cushions for air conduction. This equipment is powered with rechargeable batteries and designed for hearing testing in schools, industry, and primary care facilities. The audiometers were calibrated to ISO64, with biological calibration performed at frequent intervals. Ambient noise levels were measured with the Quest electronics model 2700 impulse sound level meter (for details see reference note 4), using slow meter response in A filter weighting. Since the human ear is not uniformly sensitive to all frequencies, this filter gives a weighted frequency response simulating the human ear.

Audiometry tests

MMC was instructed and trained at the Otorhinolaryngology Department, Audiology Unit, at the University Medical Centre of Groningen; EA and WT were instructed by MMC; at the Komfo-Anokye Teaching Hospital, the trained audiology assistant (GO) tested the participants. The protocol for the present validity and reliability study was tested before study participants were enrolled. Both ears were tested, the right ear first. Only this audiogram was used for analysis, since testing the second ear of a participant would be influenced by the experience of the first ear already tested.
Pure tone hearing level thresholds were obtained for each participant. Frequencies

were tested in the following order: 1, 2, 4, 6, 8, 0.5 and 0.25 kHz, using descending levels of intensity. Testing started with presenting a loud tone. From this level the intensity was decreased in steps of 5 dB until the tone was not heard anymore. The last tone still heard was the level of audibility of the first test run. Then the intensity was increased with 10 dB before being decreased again in steps of 5 dB, until the tone was no longer heard (the second test run). If the results of the two test runs were not identical, the process was repeated until two identical levels of audibility were found. This level was considered the hearing threshold. Thus each frequency was tested at least twice. At the Komfo-Anokye Teaching Hospital, a different protocol was used, following local practice; thresholds were measured with ascending levels of intensity, starting at 0 dB. From this level the intensity was increased in steps of 5 dB until the tone was heard. This frequency was the level of audibility of the first test run. Then the intensity was decreased with 10 dB before being increased again in steps of 5 dB, until the tone was heard again (the second test run). If necessary, the process was repeated until two identical levels of audibility were found. The same frequency order was used, and after studying the other frequencies, 1 kHz was re-tested. The portable audiometer is limited to a maximum of 100 dBHL. 'No response' would be recorded as a 105 dB hearing threshold. At least one hour, but no more than half a day (morning to afternoon), was scheduled between the first and the second test. No treatment was administered in between.

Ethics

The Committee on Human Research, Publications and Ethics, School of Medical Sciences, Kwame Nkrumah University of Science & Technology, Kumasi, approved the study protocol and the consent procedure. From all participants >12 years informed consent was obtained; parents, guardians or caretakers gave informed consent for all participants <18 years. Information was provided about the purpose of the study and the extra time it would take to participate. Prospective participants were told that neither their enrolment in the study nor their refusal or withdrawal to participate would affect their health or treatment in any way. They were informed that the tests were an evaluation necessary for the study itself, and that the tests were not done because of suspected pathology. They were offered appropriate time to consider participation. A signature or a thumbprint confirmed verbal consent, as most of the participants were illiterate. All participants were offered a small incentive (food products). Travel costs were reimbursed.

Definitions

In conventional audiometry, hearing impairment is classified by a pure-tone average of hearing thresholds over frequencies 0.5, 1, 2 and 4.0 kHz as follows: slight (16 – 25 dBHL), mild (26 – 40 dBHL), moderate (41 – 55 dBHL), moderately severe (56 – 70 dBHL), severe (71 – 90 dBHL) or profound (>90 dBHL) in the better ear [22].

An important concern about assessing hearing levels in the field is the influence of ambient noise. The American National Standards Institute (ANSI) has addressed this problem and specified maximum permissible ambient noise levels allowed in an audiometric test room to ensure that hearing thresholds obtained down to 0 dBHL will not be elevated due to masking by ambient noise. Maximum permissible ambient noise levels when testing in the 0.25 to 8 kHz frequency range, using a supra-aural earphone, are as follows: 39 dB at 0.125 kHz, 25 dB at 0.25 kHz, 21 dB at 0.5 kHz, 26 dB at 1 kHz, 34 dB at 2 kHz, 37 dB at 4 kHz and 37 dB at 8 kHz [23].

Data analysis and reporting

For the validity study as well as the intra- and inter-observer studies, Bland and Altman plots were made [24]. These plots are designed to show differences between two measurements over their range. For each participant two measurements were available for each frequency tested. The difference between these two measurements was calculated per frequency, as well as the mean of these two measurements, per frequency. Thereafter a plot was made per frequency for the validity study as well as the intra- and inter-observer studies, of the differences between the two measurements (vertical axis) against the mean of these two measurements (horizontal axis). Then the mean of all plotted differences and the standard deviation of this mean difference were calculated per test frequency, as was the percentage of measurements within a 5 and a 10 dB difference between the two tests, per frequency. One-way ANOVA was used to analyze differences between the paired measurements for the three test locations (for the intra-observer reliability study), and for the three tester-pairs (for the inter-observer reliability study) for each frequency. Data were analyzed using SPSS 16.0 for Windows®.

We used the format as reflected in the STARD statement (Standards for Reporting of Diagnostic Accuracy) to ensure appropriate and comprehensive reporting [25].

Results

Overall, the plots for the validity and reliability studies showed no consistent relation between the mean of the repeated tests and the differences. This implies that the differences in hearing level observed in the two measurements did not vary in any systematic way depending on the (mean) level of hearing. The mean difference of the paired measurements was close to zero for all of the frequencies, i.e., the first measurement did not systematically affect the second. No ceiling effects were found; all of the participants had responded before reaching 100 dBHL, the highest presentation level of the portable machine.

Ambient noise measurements

The mean ambient noise in Agogo Presbyterian Hospital was 50 dB(A) (range 49 - 53 dB: 5 measurements); the mean ambient noise in Nkawie-Toase Governmental Hospital was 47 dB(A) (range 43 - 49 dB: 12 measurements). In Komfo-Anokye Teaching Hospital no ambient noise was measured.

Validity testing, standard versus portable equipment, KATH

For the validity study, 47 participants were included; 51% of the participants were female. The age ranged from 5 to 79 years (median 36; IQR 29 to 45 years); 92% of the participants tested underwent the hearing test for the first time in their life. Mean hearing thresholds in this group (using the mean of the first and the second measurement for every participant) were 24, 28, 27, 26, 28, 34 and 32 dBHL for 0.25, 0.5, 1, 2, 4, 6 and 8 kHz respectively.

Figure 1 shows a plot of the differences between the fixed equipment and the portable equipment against their mean at 1 kHz. Such a plot was made for each test frequency.

Table 1 summarizes the differences between the fixed and the portable equipment per frequency tested (kHz). One outlier, resulting from a test session with an elderly person, was ignored. Additionally, the mean differences, the SD of the differences and the percentage of difference falling within 5 and 10 dB are presented in table 1. In brackets, results are given including the outlier. The percentage of differences falling within 5 and 10 dB also include the outlier. In the range 0.5 to 4 kHz, 96% of repeated measurements were within 10 dB. At higher and lower frequencies, differences were larger.

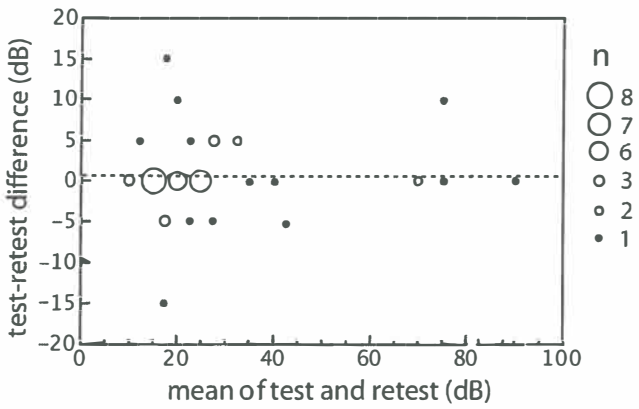

Figure 1: Plot example of test-retest differences against their mean. The dotted line in the graph is the mean difference. Test validity at 1 kHz (n=47).

Intra-observer reliability, field study

In Nkawie-Toase Governmental Hospital and Agogo Presbyterian Hospital, 51 participants were included; 60% of the participating volunteers were female. Median age was 23 years (IQR 9 – 42 years); 34% of the participants tested underwent the hearing test for the first time in their life and the same percentage for the second time. Mean hearing thresholds in this group (using the mean of the first and the second measurement for every participant) were 22, 26, 25, 22, 23, 30 and 23 dBHL for 0.25, 0.5, 1, 2, 4, 6 and 8 kHz respectively. One younger participant was withdrawn from the study during the assessments for not being able to cooperate.

Figure 2 shows a plot of the differences between the two testing sessions of the observer against their mean for 1 kHz. Such a plot was made for each test frequency. Table 2 summarizes the differences between the two measurements of the observer, per frequency. Additionally the mean differences, the SD of the differences and the percentage of differences falling within 5 and 10 dB are presented in table 2. Here, 94% of repeated measurements were within 10 dB in the range 0.25 to 4 kHz.

No significant effect of test location on the differences between paired measurements was found ($p>0.1$ for all the test frequencies), and no significant effect of previous experience with the test on the difference between paired measurements was found ($p>0.2$ for all the test frequencies).

Table 1: Test validity – differences between the fixed and the portable equipment per tested frequency (with number of subjects (n=47) in *Italics).*

Freq (kHz)	Test-retest differences (dB)											Mean of diff. (dB)	SD of diff. (dB)	± 5 dB (%)	± 10 dB (%)
	-25	-20	-15	-10	-5	0	5	10	15	20	25				
0.25	*0*	*1*	*1*	*4*	*15*	*12*	*5*	*5*	*2*	*2*	*0*	-0.1	8.4	68	87
0.5	*0*	*0*	*0*	*5*	*11*	*13*	*13*	*3*	*1*	*1*	*0*	0.5	6.6	79	96
1	*0*	*0*	*1*	*0*	*6*	*30*	*7*	*2*	*1*	*0*	*0*	0.5	4.6	92	96
2	*0*	*0*	*1*	*1*	*10*	*14*	*12*	*8*	*0*	*1*	*0*	1.8	6.5	77	96
4	*0*	*0*	*2*	*2*	*8*	*23*	*7*	*5*	*0*	*0*	*0*	-0.1	5.8	81	96
6	*0*	*1*	*2*	*4*	*10*	*16*	*7*	*4*	*2*	*1*	*0*	-0.3	8.0	70	87
8	*0*	*1*	*1*	*2*	*15*	*10*	*10*	*3*	*3*	*1*	*0*	0.3 (1.0)*	7.9 (9.0)*	75	85

*Numbers in brackets include one outlier (30 dB at 8 kHz).

Table 2: Intra-observer reliability - differences between the two measurements of one observer, per frequency, using portable equipment in the field (with number of subjects (n=50) in *Italics).*

Freq (kHz)	Test-retest differences (dB)											Mean of diff. (dB)	SD of diff. (dB)	± 5 dB (%)	± 10 dB (%)
	-25	-20	-15	-10	-5	0	5	10	15	20	25				
0.25	*0*	*0*	*0*	*2*	*12*	*23*	*12*	*1*	*0*	*0*	*0*	-0.2	4.3	94	100
0.5	*0*	*0*	*0*	*2*	*13*	*18*	*15*	*2*	*0*	*0*	*0*	0.2	4.7	92	100
1	*0*	*0*	*0*	*1*	*10*	*25*	*13*	*1*	*0*	*0*	*0*	0.3	4.0	96	100
2	*0*	*0*	*2*	*4*	*11*	*18*	*9*	*5*	*0*	*1*	*0*	-0.2	6.8	76	94
4	*0*	*0*	*0*	*5*	*12*	*14*	*16*	*1*	*2*	*0*	*0*	0.2	6.0	84	96
6	*0*	*2*	*2*	*0*	*8*	*16*	*12*	*7*	*3*	*0*	*0*	1.3	7.9	72	86
8	*0*	*2*	*3*	*5*	*10*	*15*	*7*	*7*	*0*	*1*	*0*	-1.2	8.4	64	88

Table 3: Inter-observer reliability – differences between the two measurements of an observer-pair, per frequency, using portable equipment in the field (with number of subjects (n=52) in *Italics*).

Freq (kHz)	Test-retest differences (dB)											Mean of diff. (dB)	SD of diff. (dB)	± 5 dB (%)	± 10 dB (%)
	-25	-20	-15	-10	-5	0	5	10	15	20	25				
0.25	*0*	*0*	*0*	*3*	*6*	*24*	*12*	*5*	*2*	*0*	*0*	1.5	5.6	81	96
0.5	*0*	*0*	*0*	*1*	*9*	*20*	*14*	*8*	*0*	*0*	*0*	1.8	5.1	83	100
1	*0*	*0*	*0*	*1*	*9*	*24*	*12*	*5*	*1*	*0*	*0*	1.4	5.0	87	98
2	*0*	*0*	*1*	*3*	*11*	*17*	*14*	*5*	*1*	*0*	*0*	0.7	6.0	81	96
4	*1*	*0*	*0*	*4*	*10*	*14*	*12*	*10*	*0*	*0*	*1*	1.4	7.8	69	96
6*	*0*	*2*	*2*	*1*	*10*	*13*	*9*	*9*	*4*	*0*	*0*	1.3 (2.5)*	8.5 (10.3)*	62	81
8	*1*	*0*	*1*	*7*	*13*	*11*	*11*	*5*	*2*	*1*	*0*	-0.4	8.3	67	91

*Numbers in brackets include two outliers (30 dB and 35 dB at 6 kHz).

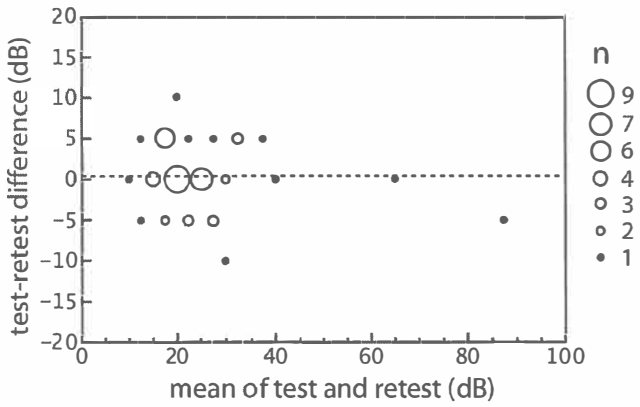

Figure 2: Plot example of test-retest differences against their mean. The dotted line in the graph is the mean difference. Intra-observer reliability at 1 kHz (n=50).

Inter-observer reliability, field study

In Nkawie-Toase Governmental Hospital and Agogo Presbyterian Hospital, 52 participants were included; 50% of the participants were female, median age 27 years (IQR 16 - 47 years); 70% of the participants did the hearing test for the first time. Mean hearing thresholds in this group (using the mean of the first and the second measurement for every participant) were 20, 23, 23, 22, 25, 28 and 24 dBHL for 0.25, 0.5, 1, 2, 4, 6 and 8 kHz respectively. As for validity and intra-observer reliability studies, plots were made of the differences between the two testing sessions against their mean for each frequency. Table 3 summarizes the differences between the two measurements of an observer-pair, per frequency; here, two outliers were ignored. These outliers resulted from a test session with a 50 year old and a 9 year old participant. In addition, the mean differences, the SD of the differences, and the percentage of differences falling within 5 and 10 dB are presented in table 3. In the range 0.5 to 4 kHz, 96% of recordings were within 10 dB. In brackets, results are given including the outliers. The percentage of differences falling within 5 and 10 dB also include the outliers.

No significant difference was observed between the three different observer pairs in the differences of the paired measurements ($p>0.3$ for all of the test frequencies), and no significant effect of previous experience with the test on the difference between paired measurements was found ($p>0.1$ for all the test frequencies).

Discussion

Though pure-tone audiometry using portable equipment has been used in rural Africa, this is the first formal reported evaluation of audiometry using portable equipment in field conditions in less affluent countries. This study shows that the validity and the reliability of the portable audiometer were satisfactory, especially in the 0.25 to 4 kHz frequency range. At 0.25, 0.5, 1, 2, and 4 kHz intra-observer test-retest repeatability was within 10 dB for at least 94% of the measurements, and inter-observer test-retest repeatability was within 10 dB for at least 96% of the measurements. At higher frequencies (6 and 8 kHz), measurements were less reliable. In clinical conditions, a pure-tone threshold measurement at a single frequency has a 90% chance to be repeated within 10 dB of the first measurement, assuming that no real change in hearing thresholds has occurred [20]. Though formal reliability studies of field portable audiometry have not been reported, Robinson (1991) examined audiometric repeatability in an industrial setting, in which the hearing level threshold at a single frequency was found to have a chance of 90% to be repeated within 10 dB, even after an interval of several years [26]. As part of a large national epidemiological study, Lutman et al. (1989) compared a manual and a computer controlled method [27]. The thresholds measured were at 0.5, 1, 2 and 4 kHz. Standard deviations of the test-retest differences with a 2 to 3 years interval were provided, and were similar to those found in our field study. Ambient noise levels were not measured. Although the test conditions, which were not ideal, were close to the current study, the time lapse between tests was considerably longer. With more time lapse between tests, SDs will become larger [28]. Authors of both studies concluded that shifts were due to random measurement error rather than actual shifts in hearing thresholds.
In another study where a group of individuals with sensorineural hearing impairment and a group of healthy individuals were tested twice, within two weeks, in standard sound proof conditions, SDs were lower than in the current field study [29]. The short interval between the tests was similar to this field study. The sound-proof conditions were not comparable. SDs in the normal and the cochlear-impaired group were equivalent. Table 4 and figure 3 show the SDs of the described studies together with those of the current study. For all studies, highest SDs were seen in the 6 and 8 kHz frequencies. Generally, reliability of conventional pure-tone audiometry decreases at higher frequencies. At these frequencies, a different positioning of the earphones has a higher influence on changes in the ear canal resonance, and therefore results in a higher variability [30,31].

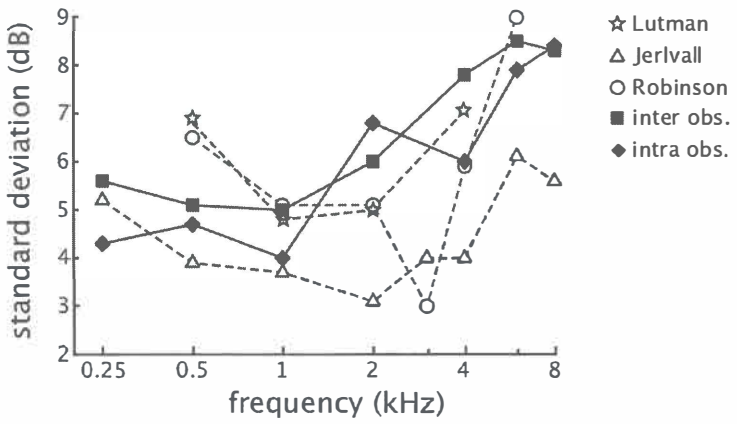

Figure 3: SDs (dB) of the in the text described inter-observer studies together with the SDs of the present intra- and inter-observer reliability study.

The reliability of hearing level thresholds measured with circum-aural earphones in the 0.5 to16 kHz frequency range was evaluated by Schmuziger et al [18]. At each individual frequency in the 0.5 to 8 kHz test frequency range, test-retest repeatability was within 10 dB for at least 99% of the measurements. SDs were not provided. That study was performed in sound-proof conditions, and this probably explains why the repeatability in the current field study was less. Besides the ambient noise levels, other factors like differences in educational level might in part explain differences in study results. The mean age of the participants was similar to the mean age of the participants in our study.

This study has several limitations. The fairly high levels of ambient noise measured in the current field studies may have caused variability to become larger. Ambient noise levels were variable within one test session but also between test sessions, so the ambient noise did not cause a systematic error and therefore does not present a source of bias, but merely caused a reduction in precision. This means that levels of hearing impairment might be overestimated, but that changes over time can be reliably detected. Secondly, although the influence of this factor was kept as small as possible by starting with a short period of practice, the differences in experience of study participants might have affected test results. For intra-observer testing, 66% of the participants had done the test before; for validity and inter-observer testing only 8% and 30% respectively had done the test before. Although statistically we could not show this, and the Bland and Altman plots did not confirm a learning curve from the first to the second test, lack of experience with the test can be one of the

causes of the observed difference in test validity, intra-, and inter-observer reliability results. Apart from ambient noise and experience, variation in response strategy, motivation, attention and intelligence of the participants may influence test reliability. Most participants were young, and they clearly had difficulty concentrating.

Table 4: SDs (dB) of repeated measurements per frequency in the different studies described in the text, compared to the results of the present study.

	Lutman et al.	Robinson et al.	Jerlvall et al.	Present study; intra-observer reliability	Present study; inter-observer reliability
No. of subjects	120	356	20	50	52
Time lapse	2-3 years	13 months	2 weeks	half a day	half a day
Study conditions	industry - clinical	industry	laboratory	field	field
0.25 (kHz)	-	-	5.2	4.3	5.6
0.5	6.9 (dB)	6.5	3.9	4.7	5.1
1	4.8	5.1	3.7	4.0	5.0
2	5.0	5.1	3.1	6.8	6.0
3	-	3.0	4.0	-	-
4	7.1	5.9	4.0	6.0	7.8
6	-	9.0	6.1	7.9	8.5
8	-	-	5.6	8.4	8.3

Conclusion

WHO recommends early detection and intervention as key to preventing and managing hearing impairment, and also states that most effective interventions can be done at the primary level of health care (reference note 5). The results in this study show that the portable audiometer can serve the call to action, as it can be well operated in the field by different observers with minimal training, which is useful in daily practice. To pick up changes >10 dB over time as a result of interventions, audiometric check-ups in the field with portable equipment are feasible, and reliable, especially in the 0.25 to 4 kHz range. As AIO affects frequencies higher than 4 kHz first, this study implies that early detection of AIO with portable audiometry in the field is limited. Absolute deviations from reference values should be interpreted with care, especially in relation to ambient noise. For this, studies comparing field and clinical audiometry are needed.

Author contributions

Designed the study: YS, MMC, WAN, GKA, KMA, TSW. Performed the experiments: MMC, WT, EA, GO. Analyzed the data: WAN, MMC, PUD, HPW. Wrote the paper: WAN, MMC, YS, GKA, KMA, WT, EA, GO, PUD, HPW, TSW.

Acknowledgments

We thank all participants and the staff of the three hospitals involved, for their help in the study. We thank Mimi Zeiger for her expert advice on the draft of the manuscript.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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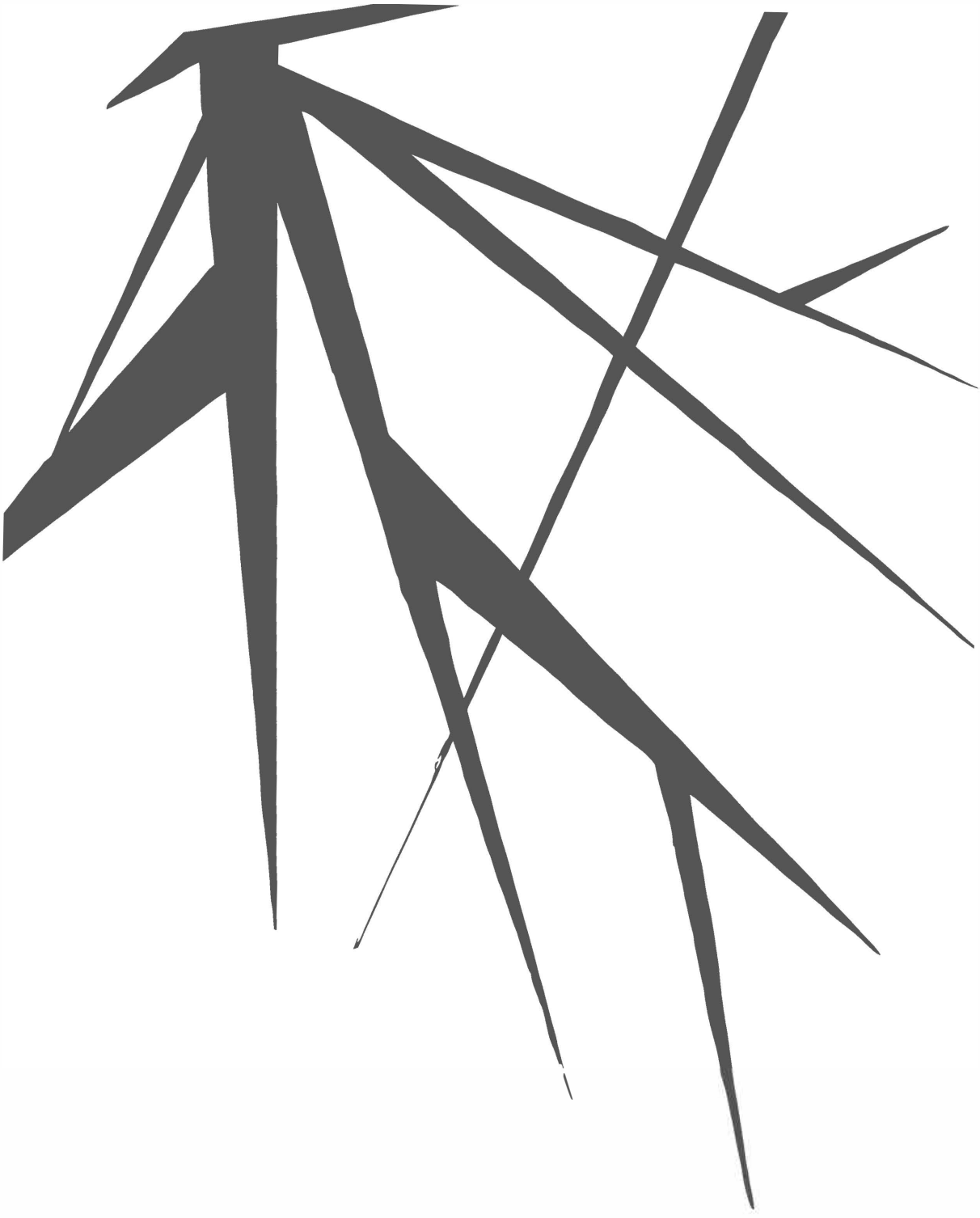

CHAPTER 6

Susceptibility to Buruli Ulcer disease and variations in SLC11A1, MBL2, VDR genes and serum vitamin D concentrations: a case-control study in Ghana

Willemien A. Nienhuis
Eveline van der Veer
Cleo C. van Diemen
K. Mohammed Abass
Wilson Tuah
William A. Thompson
Peter C. Awuah
Joseph M Frimpong
Justice Abotsi
Victoria Ahiable
Karin Koerts
Bahram Sanjabi
Pieter van der Vlies
Ymkje Stienstra
Tjip S. van der Werf

Manuscript *submitted*

Abstract

Background Serological studies show that only a small proportion of *Mycobacterium ulcerans* infected individuals develop clinical disease (Buruli ulcer disease). The genetic and environmental factors determining progression to overt *M ulcerans* disease are likely to be complex.

Methodology/principal findings In a case-control study we sought confirmation of our earlier finding that susceptibility to *M ulcerans* disease is associated with certain polymorphisms in the SLC11A1 gene. We also investigated polymorphisms in the MBL2 and the VDR genes because of their association with tuberculosis and leprosy. Because of its association with tuberculosis, serum 25-hydroxyvitamin D concentrations were measured to explore a possible relation to disease status. 179 Ghanaian *M ulcerans* patients and 180 age, gender and ethnicity matched controls were included. Susceptibility to develop *M ulcerans* disease appeared not associated with any of the polymorphisms studied. Serum vitamin D concentrations were lower in patients, compared to controls (65.9 vs 73.0 nmol/L; $p<0.001$).

Conclusions/Significance In this sample size with a different ethnic background (i.e., other tribal origins than the previous study), we were unable to confirm the association with SLC11A1 gene polymorphisms for *M ulcerans* disease. We found a significantly lower vitamin D blood concentration in patients as compared to control subjects. This finding might either reflect increased consumption of vitamin D by cells of the immune system at the site of infection, or less likely, decreased exposure to sunlight resulting from covered skin or inability to participate in farming and other out-door activities, due to *M ulcerans* disease.

Author summary

Buruli ulcer disease (*Mycobacterium ulcerans* infection) is a disabling, neglected disease, emerging around the world and notably in sub-Saharan Africa. Like tuberculosis and leprosy, environmental and inherited factors determine whether or not clinical disease follows after infection. We studied three genes, associated with tuberculosis and leprosy, among Buruli ulcer patients and matched healthy controls. We could not confirm our earlier finding of a strong association between polymorphisms in the SLC11A1 gene and Buruli ulcer, nor for the other genes (MBL2 and VDR) studied. The majority of our Buruli ulcer patients were immigrants from Northern Ghana who had farms in the Buruli ulcer-endemic area where our study was conducted. Perhaps a difference in genetic background compared to the previous study explains why this time we found no genetic associations.

Vitamin D is important in the defense against mycobacterial infection such as tuberculosis. Vitamin D is essential for the production of the anti-mycobacterial peptide cathelicidin. We found lower serum vitamin D concentrations in Buruli ulcer patients compared to control subjects. We speculate that consumption of vitamin D at the site of infection might explain this. This new finding could be important in finding ways to improve disease management.

Introduction

Buruli ulcer disease (*Mycobacterium ulcerans* infection) is an indolent, ulcerating and devastating disease of the skin [1,2]. If not diagnosed and treated early, it carries the risk of leaving patients with severe and permanent functional limitations [3]. It is the third most common human mycobacteriosis in immunocompetent individuals after tuberculosis and leprosy [4]. In the last decades Buruli ulcer disease (BUD) is emerging in dramatic numbers in sub-Saharan Africa. With an incidence in some areas surpassing that of tuberculosis and leprosy, Buruli ulcer has an immense social and financial impact on individuals and on the public health system [5]. The WHO selected BUD as one of nineteen neglected tropical diseases [6]. Having its endemic places mostly in tropical countries in remote areas, Buruli ulcer is called a disease of the poor. Children are more often affected than adults. The mode of transmission and reservoir of the disease are yet unknown [7], but are not restricted to the tropics alone; Australia being the second continent burdened by this disfiguring disease [8]. There is no evidence for important person-to-person transmission.

Serological studies show that only a small proportion of *M ulcerans* infected individuals develop clinical disease [9,10]. For tuberculosis and leprosy it is known that genetic predisposition plays an important role in the chance of developing disease [11-14]. Support for this idea came from observations of familial clustering, high concordance rates in identical twins and clear racial differences in the risk of developing disease. Like in tuberculosis and leprosy, the genetic influence on developing disease is likely to be complex, with several different genetic polymorphisms playing each a limited role to explain the variance observed in the population at risk. Earlier, our group reviewed possible genetic host susceptibility factors for BUD, relevant in other mycobacterial diseases [15]. Based on a limited sample of cases and healthy control subjects living in an endemic region in Ghana, it was shown that carrying the heterozygous GA genotype of the D543N polymorphism in the SLC11A1 (formerly Nramp1) gene may be associated with an increased risk of developing BUD [16]. In the present study we intended to confirm these findings. The SLC11A1 gene encodes a transport protein expressed in the membrane of late endosomes mainly in macrophages, regulating antimicrobial activity. The exact mechanism by which microbial replication is blocked is not entirely clear. Several association studies have indicated that polymorphisms in this gene may be associated with clinical tuberculosis and leprosy [17,18], as well as leprosy type [19].

We selected two other candidate genes from the published review that have not been studied before in relation with BUD, i.e., the mannose binding lectin (MBL) gene and the vitamin D receptor (VDR) gene. MBL is a liver derived complement activating serum protein [20], which can bind surface structures of several clinically important bacteria, yeast and viruses. It initiates complement activation and phagocytosis and induces inflammatory cytokine responses. In general, MBL deficiency, which is mainly determined by single nucleotide polymorphisms, is common en predisposes to various infections [21]. Low serum MBL levels may also confer protection against some intracellular parasites like leishmaniasis, leprosy and tuberculosis [22-25]. The high prevalence of infections caused by intracellular pathogens in people of African descent may explain the elevated frequency of alleles causing low levels of MBL as compared to people from European descent [26,27].

Vitamin D enhances antimycobacterial activity in *in vitro* systems and restricts growth of *M tuberculosis* [28]. The effect of the active metabolite (1,25-dihydroxyvitamin D), is exerted by interaction with the intranuclear vitamin D receptor [29,30]. Epidemiological evidence suggests there is a relation between vitamin D deficiency, as well as polymorphisms in the VDR gene, and susceptibility to tuberculosis [31-34]. In a study that assessed the potential of vitamin D suppletion in tuberculosis

patients, VDR polymorphism seemed to be associated with time to sputum culture conversion [35]. VDR gene polymorphism has also been found to be associated with leprosy type (tuberculoid versus lepromatous) [36].
The objective of this study was to investigate association of BUD with SLC11A1, MBL and VDR gene polymorphisms, as well as with serum vitamin D concentrations, in patients and healthy controls coming from two regions in Ghana highly burdened with BUD. We hypothesized that the type of lesion, extent of disease, and response to antimicrobial therapy also may be determined by genetic host factors.

Methods

Participants: case ascertainment and matched control selection

Blood samples were collected and demographic and clinical information was obtained from patients who were participating in a randomised controlled trial to compare two different antimicrobial regimens [37]. This trial was conducted in two hospitals located in the Ashante Region of Ghana. BUD patients were included from April 2006 to January 2008. Participants were aged ≥5 years, had early, limited and IS2404 PCR confirmed disease and were followed for one year from start of treatment. Detailed inclusion criteria for the randomised trial can be found on ClinicalTrials.gov, identifier NCT00321178. Patients that had clinical disease that could not be PCR confirmed were also treated and handled according to the same protocol. These were included in the present study too, as well as PCR confirmed patients that were not randomised for different reasons, but had blood samples taken.
In June 2008, one or two matched community controls were enrolled per patient. Control subjects were preferably befriended with, and living close by the house of the BUD patient. We matched for age, as BUD is known to have a bimodal age distribution, with an increased incidence in young persons and in the elderly [38,39]. We matched for gender as some studies, as well as our antibiotic study population, show that women are more prone to develop BUD [37,40-42]. During patient recruitment for the antibiotic trial we were struck by the fact that the majority of BUD patients were not Akan, the dominant ethnic group where the study was conducted [37]. Therefore, we matched according to two major ethnic groups; Akan being individuals from the region where the study was conducted; and other, consisting of individuals mainly belonging to different ethnic groups from Northern

Ghana. If a study participant's parents were from different descent, we preferably chose the descent of the mother to select the matched control.

Ethical considerations

The protocol and consent forms were approved by the Committee on Human Research, Publication and Ethics of the Kwame Nkrumah University of Science and Technology and the Komfo Anokye Teaching Hospital, Kumasi (CHRPE/07/01/05) and by the Ethical Review Committee of Ghana Health Services (GHS-ERC-01/01/06). Informed consent and assent was given by all participants aged ≥12 years and/or from parents, care takers or legal representatives of participants ≤18 years.

Genotyping

Blood samples in EDTA tubes for buffy coat were centrifuged within 24 hours after collection, stored at -20°C and sent in frozen condition from Ghana to the University Medical Center Groningen, the Netherlands, until processed. Genomic DNA was extracted using QiaAmp 96 blood kit (Qiagen).

We genotyped four polymorphisms in the SLC11A1 gene: rs59823161 (3'UTR TGTG ins/del); rs17235409 (D543 G/A); rs3731865 (INT4 G/C); and a $(CA)_n$ microsatellite in the immediate 5' region of the gene. The 5'$(CA)_n$ microsatellite analysis was done using the Applied Biosystems 3730 capillary sequencer; with fluorescent primer 5'-ACTCGCATTAGGCCACAGAG and 5'-TTCTGTGCCTCCCAAGTTAGC. Normal genotype was designated "200" and variant genotypes (202, 204) were pooled as "other". We genotyped two polymorphisms on the MBL2 gene as well. Both are known to cause inter-individual variations in serum MBL levels. One of these, rs1800451, is a point mutation on exon 1 at codon 57. Wild type is denoted as allele A. The minor C-allele is predominantly found in African populations. The second polymorphism, rs7096206, a base-pair substitution in the promoter region (G -› C at position 221), changes the (most common) allele called Y into allele X. The VDR single nucleotide polymorphisms genotyped were rs2228570 (Fok1), a C-›T base change in exon 2; rs731236 (Taq1), a silent T-›C base change at codon 352 in exon 9; 1544410 (Bsm1), a T-›C base change in intron 8; and rs7975232 (Apa1), a G-›T base change in intron 8. TaqMan MGB-probes and primers were obtained through the Applied Biosystems assay-by-design service.

Vitamin D serum concentration

Blood samples in clotted blood tubes for serum were cooled until centrifuged within 24 hours after collection, then stored at -20°C, and sent in frozen condition from

Ghana to the University Medical Center Groningen, the Netherlands, until processed. Serum concentration of 25-hydroxyvitamin D, the primary indicator of vitamin D status, was determined in duplex for every sample by a radioimmunoassay (DiaSorin, Stillwater, MN) [43]. The mean of these two results was used for the analyses.

Statistical analysis

Baseline characteristics of the patient and the control group were compared using Mann-Whitney U, Pearson Chi-Square and Fisher's Exact test as appropriate. Genotype and allele frequencies of individual polymorphisms were determined by direct counting and expressed as percentages. Genotyping was checked by assessing whether polymorphisms of controls were in Hardy-Weinberg equilibrium, using a χ^2 test with one degree of freedom (df). Association between BUD and the polymorphisms was studied using cross tabulation with χ^2 tests. The odds ratios (OR) and 95% confidence intervals (CI) were determined using binary logistic regression. To adjust for potential confounding: age, gender, study site, ethnicity, and profession were included in the model. Because association studies have linked farming activities with the chance of acquiring BUD, profession was divided in *farming* and *other*.

To study whether type of lesion (nodule, plaque, ulcer, edema), lesion category (category I; lesions < 5 cm diameter, or category II/III; lesions >5 cm and multiple lesions) or response to therapy (healing or failure) were associated with certain polymorphisms, cross tabulation was used with χ^2 tests. Heterozygote and homozygote variant alleles were combined when individual groups were too small to perform the test, with Fisher's Exact as appropriate. For these multiple testing, the significance level was adjusted according to Bonferroni.

Comparison of vitamin D levels between patients and controls, and between the different VDR polymorphisms, was performed by Mann-Whitney U tests for two independent samples and by Kruskal-Wallis tests for more than two categories.

The association analyses were done with SPSS (version 16.0; Chicago, IL, USA). Hardy-Weinberg equilibrium testing was performed in MS Excel. Unless stated otherwise, p-values less than 0.05 were considered significant.

Results

Of the BUD patients, 151 were participants in the randomised antibiotic trial. The other 28 patients were not randomised but received standard antibiotic treatment, and were handled according to the same protocol. Of the 179 patients, 163 (91%) had

confirmed disease; 156 were at least PCR-confirmed; 7 were confirmed otherwise (Ziehl-Neelsen staining for acid-fast bacilli, culture and/or histopathology). 12 patients provided two controls and for 11 patients no control could be selected, which resulted in a total of 180 controls. Table 1 shows the baseline characteristics of the 179 BUD patients and the 180 controls included in the analysis. These characteristics were similar for both groups.

Table 1: Baseline characteristics of the Buruli ulcer disease patient group and the control group

	patients (n=179)	**controls (n=180)**	**p***
median age in years (IQR)	12 (8-20)	13 (9-21)	0.16
male sex (%)	62 (35%)	63 (35%)	0.94
ethnicity (% Akan vs other ethnic groups)	54 (31%)	58 (32%)	0.73
profession (% farmer vs other and pupils)	48 (27%)	49 (28%)	0.91
study site (% Nkawie vs Agogo)	52 (29%)	51 (28%)	0.88
HIV infected individuals (n)	4 (2%)	1 (0.6%)	0.37

* Mann-Whitney U test for continuous and Pearson Chi-Square test for categorical data, with Fisher's Exact test for tables where >20% of the expected frequencies were <5

The selected polymorphic markers and their genotype frequency distributions are shown in table 2, 3 and 4. There was no suggestion of gross genotyping errors, as all polymorphisms in the control group were in Hardy-Weinberg equilibrium. Missing data were less than 6% for all polymorphisms genotyped. None of the polymorphisms of the SLC11A1, MBL and VDR genes studied were significantly associated with having BUD. Results did not change when only PCR confirmed patients were included, or when HIV-infected individuals were excluded from the analysis (data not shown).
When using Bonferroni correction for multiple testing, the polymorphisms were not associated with stage of disease, with type of lesion, or with response to treatment (data not shown).
In 345 individuals vitamin D concentrations could be obtained from serum, 14 individuals (12 patients and 2 controls) had a missing value. Table 5 shows median 25-hydroxyvitamin D serum concentrations for patients and for controls, grouped by gender. Serum vitamin D concentrations were different ($p<0.001$) in patients and controls, with for patients a median of 65.9 (25-75th percentile: 55.9-77.6) nmol/L and for controls 73,0 (63.1-84.1) nmol/L. In males the difference was larger than in females.

Table 2: SLC11A1 genotype frequencies in Buruli ulcer patients and in controls

	Buruli ulcer patients (n=179)	controls (n=180)	crude odds ratio (95%CI)[a]	adjusted odds ratio (95%CI)[b]
INT4				
G/G	151 (87%)	157 (91%)	1	1
G/C	21 (12%)	15 (9%)	1.46 (0.72-2.93)	1.45 (0.72-2.94)
C/C	1 (1%)	0 (0%)	NA	NA
missing	6	8		
D543N				
G/G	148 (85%)	152 (86%)	1	1
G/A	26 (15%)	23 (13%)	1.16 (0.63-2.13)	1.17 (0.64-2.15)
A/A	1 (1%)	1 (1%)	1.03 (0.06-16.6)	1.08 (0.07-17.5)
missing	4	4		
3'UTR				
TGTG ins/ins	89 (51%)	94 (55%)	1	1
TGTG ins/del	74 (43%)	67 (39%)	1.17 (0.75-1.81)	1.14 (0.73-1.79)
TGTG del/del	10 (6%)	11 (6%)	0.96 (0.39-2.37)	0.98 (0.40-2.44)
missing	6	8		
5'(CA)n				
200/200	94 (56%)	106 (62%)	1	1
200/other	65 (38%)	58 (33%)	1.26 (0.81-1.98)	1.21 (0.77-1.92)
other/other	10 (6%)	8 (5%)	1.41 (0.53-3.72)	1.53 (0.56-4.22)
missing	10	8		

[a]Odds ratios are for comparison with the most common genotype for each polymorphism.
[b]Odds ratios via binary logistic regression analysis with gender, age, ethnicity and profession as covariates in the model.

Table 3: MBL genotype frequencies in Buruli ulcer patients and in healthy controls

	Buruli ulcer patients (n=179)	controls (n=180)	crude odds ratio (95%CI)[a]	adjusted odds ratio (95%CI)[b]
codon 57				
WT/WT	84 (49%)	86 (50%)	1	1
WT/M	80 (46%)	72 (42%)	1.14 (0.73-1.76)	1.12 (0.72-1.75)
M/M	9 (5%)	15 (9%)	0.61 (0.26-1.48)	0.63 (0.26-1.54)
missing	6	7		
promoter (-221)				
YY	134 (77%)	132 (75%)	1	1
Y/X	39 (22%)	42 (24%)	0.92 (0.56-1.51)	0.94 (0.56-1.55)
X/X	2 (1%)	2 (1%)	0.99 (0.14-7.10)	0.98 (0.14-7.12)
missing	4	4		

Y = common allele of -221 polymorphism
X = less frequent allele of -221 polymorphism
WT = common allele of 57 codon polymorphism
M = mutant allele for 57 codon polymorphism
[a]Odds ratios are for comparison with the most common genotype for each polymorphism.
[b]Odds ratios via binary logistic regression analysis with gender, age, ethnicity and profession as covariates in the model.

Discussion

This study does not confirm the strong association of carrying one of the SLC11A1 polymorphisms with susceptibility for *Mycobacterium ulcerans* infection, as shown earlier; nor do the MBL or VDR polymorphisms show an association with infection. We show that vitamin D serum concentration is associated with *M ulcerans* infection. We speculate that the reduced 25-hydroxyvitamin D serum concentrations that we found in patients compared to control subjects, is related to consumption of vitamin D at the site of infection. In tuberculosis, formation of activated vitamin D (1,25-dihydroxyvitamin D) from 25-hydroxyvitamin D upregulates the production of cathelicidin, an antimicrobial peptide [30,44-47]. The vitamin D mediated antimicrobial activity against intracellular *M tuberculosis* was found to be dependent on cathelicidin, via activation of toll-like receptors (TLRs) on human monocytes and macrophages [48]. After infection with *M tuberculosis*, TLR activation results in conversion of 25-hydroxyvitamin D into 1,25- dihydroxyvitamin D, within human macrophages, at the site of infection [49]. Blockade of LL-37, the active peptide form of cathelicidin, results in increased growth of *M ulcerans* in human epidermal keratinocytes [50].
It is probable that *M ulcerans*, like *M tuberculosis*, activates TLRs, resulting in the formation of cathelicidin via conversion of vitamin D. Another cause of lower serum vitamin D concentrations in patients might be less sunlight exposure, resulting from the stigma and functional limitations known to be associated with Buruli ulcer disease [51,52]. Thirdly, a pre-existing low vitamin D concentration – either as a result of reduced dietary intake, or less exposure to sunlight, might result in less protective immunity against *M ulcerans*, with a larger chance of developing clinical disease after infection. The very small difference in median serum vitamin D concentrations between patients and controls, and the overall high vitamin D concentrations make this explanation less probable.
We realized that the different time period in which vitamin D serum samples were taken could have induced bias. Samples from patients were taken throughout the year, while samples from controls were taken in the month of June, during rainy season. The relatively cloudy weather in the rainy season would have favoured lower, not higher vitamin D concentrations; the bias would rather be in the opposite direction than what we found, and it is therefore not very likely that differences in sampling time between cases and controls explain our findings.

Table 4: VDR genotype frequencies in Buruli ulcer patients and in healthy controls

	Buruli ulcer patients (n=179)	controls (n=180)	crude odds ratio (95% CI)[a]	adjusted odds ratio (95%CI)[b]
FokI				
C/C	104 (61%)	114 (66%)	1	1
C/T	64 (38%)	51 (30%)	1.38 (0.87-2.17)	1.36 (0.86-2.17)
T/T	1 (1%)	7 (4%)	0.16 (0.02-1.29)	0.15 (0.02-1.21)
missing	10	8		
TaqI				
T/T	93 (55%)	107 (62%)	1	1
T/C	69 (40%)	56 (32%)	1.42 (0.91-2.22)	1.33 (0.84-2.10)
C/C	9 (5%)	10 (6%)	1.04 (0.40-2.66)	1.04 (0.40-2.67)
missing	8	7		
BsmI				
T/T	77 (46%)	89 (51%)	1	1
T/C	81 (48%)	67 (39%)	1.40 (0.90-2.18)	1.38 (0.88-2.17)
C/C	11 (6%)	17 (10%)	0.75 (0.33-1.69)	0.75 (0.33-1.70)
missing	10	7		
ApaI				
T/T	91 (53%)	79 (46%)	1	1
G/T	69 (40%)	77 (44%)	0.78 (0.50-1.21)	0.82 (0.53-1.29)
G/G	11 (7%)	18 (10%)	0.53 (0.24-1.19)	0.53 (0.23-1.20)
missing	8	6		

[a]Odds ratios are for comparison with the most common genotype for each polymorphism.
[b]Odds ratios via binary logistic regression analysis with gender, age, ethnicity and profession as covariates in the model

Table 5: Median 25-hydroxyvitamin D serum concentration (nmol/L)

	patients (n=167)	controls (n=178)	p[a]
median (nmol/L)	65.9	73.0	<0.001
25-75th percentile	55.9 - 77.6	63.1 - 84.1	
female	65.9 (n=111)	71.4 (n=116)	0.028
25-75th percentile	54.7 - 78.1	59.8 - 81.4	
male	65.9 (n=56)	76.9 (n=62)	<0.001
25-75th percentile	58.6 - 76.8	67.9 - 93.5	

[a]Mann-Whitney U test for two independent samples

Contrary to our earlier study [16], the present study could not confirm an important share of the SLC11A1 D345N polymorphism in acquiring clinical *M ulcerans* disease. Both studies were of the same sample size. In addition to matching for age and gender like the former study, in the present study we matched for ethnicity as well. As 75% of the Buruli ulcer patients that were included in the antimicrobial trial were from northern ethnic origin while the study took place in the centre of Ghana [37], we aimed at preventing confounding as a result of background genetic differences.As genetic studies in African populations are challenging because genetic information is

highly diverse within confined areas, confounding as a result of ethnic differences in our study population cannot be excluded [53]. We matched according to two major ethnic groups - *Akan* and *other*. As *other* consisted of over 13 ethnic groups -mainly from the northern part of Ghana, this group was probably still diverse in genetic background. However, separate analysis of only *Akan* individuals, as the former study included probably mainly *Akans*, did not change results (data not shown).
Susceptibility to develop Buruli ulcer neither seems to be related to MBL and VDR polymorphisms in our population. The median serum vitamin D concentration was around 70 nmol/L. Possibly, VDR polymorphisms only play a major role when vitamin D serum concentrations are lower. In a study that looked at serum vitamin D concentrations in Asian individuals that migrated to London, VDR polymorphisms influenced susceptibility for tuberculosis only in the subgroup with deficient or undetectably vitamin D serum concentrations [31].
A limitation of the present study is the unpowered sample size. An association with the polymorphisms therefore cannot be excluded. As in the former study a significant association with one of the SLC11A1 polymorphisms was found with a relatively small sample size, we decided the sample size would be high enough for reproduction. Higher samples sizes are difficult to obtain for *M ulcerans* infection. Studies like this are nevertheless important as results might add to developing ways of preventing or managing disease. Further analysis of the mechanisms of action and the downstream cellular pathways of susceptibility and resistance genes of the host, may lead to new approaches to control disease. Likewise, it will be helpful in identifying individuals in endemic areas that have increased risk to develop disease, giving them the opportunity to protect themselves against behavioural risks and to be aware of, and recognize *M ulcerans* infection in an early stage. In these individuals other strategies to combat disease, like a newly produced vaccine, could be considered when living in an endemic area [54].

In conclusion, no appreciable influence of carrying one of the SLC11A1, MBL and VDR polymorphisms was found on developing Buruli ulcer disease. Buruli ulcer patients had significantly lower serum vitamin D concentrations than controls, which we relate to local activation and consumption of vitamin D by cells of the immune system, possibly resulting in production of the antimicrobial peptide cathelicidin. Insight in the role of susceptibility genes and further analysis of the role of vitamin D, cathelicidin and other antimicrobial peptides is important to increase our understanding in the pathology and evolution of this disabling disease, aiming for novel tools to combat *Mycobacterium ulcerans* infection.

Contributors

TSvdW, YS and EvdV supervised the study. TSvdW, YS, WAN, PvdV and EvdV designed the study. WAN coordinated the study. WAN, KMA, WT, MrF, JA, and VA were responsible for patient and control screening and enrolment. KMA, WT, JA and VA provided patient care and requested informed consent from participants, participants' parents, or legal representatives, and collected the clinical and laboratory data. WAT and PCA gave expert advice. EvdV and KK were responsible for vitamin D serum concentration measurements. BS, PvdV and CCvD were responsible for the laboratory work on genetics. WAN and CCvD did the statistical analyses. TSvdW, EvdV, CCvD, PvdV, YS and WAN contributed to the interpretation of the results and the writing and critical review of the report. All authors have seen and approved the final version of the report. WAN is currently at the Department of Internal Medicine, Medical Center Leeuwarden, the Netherlands.

Acknowledgments

Our greatest thanks goes out to all study participants involved in the trial. We thank all staff, health personnel and community workers that were involved, and Kingsley Asiedu, Global Buruli Ulcer Initiative, WHO, Geneva, Switzerland. Elvira Oosterom was of invaluable help in the genetic work, but could not help finish the manuscript.

Funding

This work was supported by the European Union (EU FP6 2003-INCO-Dev2-015476), the JK de Cock stichting and Stichting Fonds Doctor Catharine van Tussenbroek. The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report, or in decisions about submission of results for publication. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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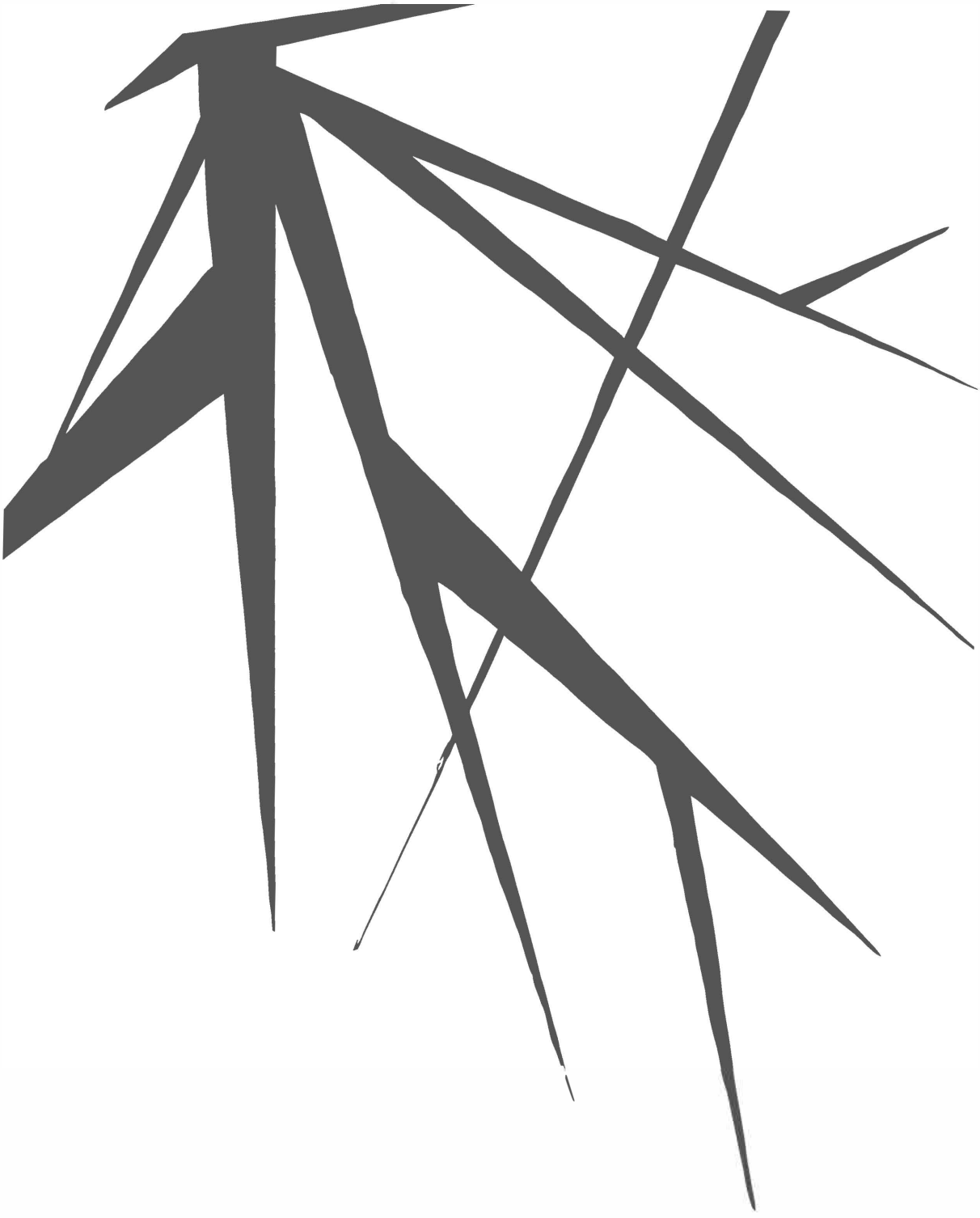

CHAPTER 7

Summary

Buruli ulcer disease, a neglected tropical ulcerating skin disease caused by *Mycobacterium ulcerans* [1,2], is denoted tool-deficient by the WHO, as it lacks cost-effective control methods [3]. This thesis focused on the effectiveness of drug therapy to control infection. Chapter 1 provided a brief introduction into the history of treatment for Buruli ulcer disease, in which surgery dominated [4]. Disappointing results of chemotherapy were poorly understood, as in laboratory experiments antibiotics appeared effective against the causative organism *Mycobacterium ulcerans*. The clinical effectiveness of combined therapy with streptomycin and rifampicin – with or without surgery, as advised by WHO from 2004 on [5], was not studied in a well-designed clinical trial with robust follow-up and pre-defined clinical end-points [6]. Chapter 1 described the scope of the thesis and outlined the hypotheses that were tested in the following chapters.

In chapter 2 we unambiguously showed the effectiveness of antibiotics for the treatment of Buruli ulcer disease, defined as clinical cure at time point 52 weeks after start of treatment [7]. In a non-inferiority design, we compared standard streptomycin and rifampicin for 8 weeks with a schedule of rifampicin for 8 weeks and streptomycin for only 4 weeks, with a switch to oral clarithromycin for a further 4 weeks. Of the 154 participants with early, limited Buruli ulcer lesions that were included in the trial, 151 were randomized. Four were lost to follow-up before the primary end-point –complete healing at one year after start of treatment – was reached; but these four participants had healed lesions at the time of their last assessment, and were included in the analysis. Randomization was done remotely in the Netherlands with help of cell-phone text messaging. Five participants needed extensive surgery, and of six participants lesions were not healed at time point one year. 73 (96%) participants in the 8-week streptomycin group and 68 (91%) participants in the 4-week streptomycin plus 4-week clarithromycin group had healed lesions at one year (odds ratio 2·49, 95% CI 0·66 to infinity; p=0·16, one-sided Fisher's exact test). No participants had lesion recurrence at 1 year. Five participants received skin grafting. Few side effects were reported; three participants had vestibulotoxic events, three reported abdominal discomfort, one developed an injection abscess and two developed an abscess close to the initial lesion, which was incised and drained.
It can be concluded from this study that antimycobacterial treatment for *Mycobacterium ulcerans* infection is highly effective in early, limited disease. As both study arms had similar efficacy, the number of injections of streptomycin can be reduced, by switching to oral clarithromycin after 4 weeks.

In chapter 3 we discussed the complex bidirectional pharmacokinetic interactions involved when combining rifampicin and clarithromycin in the treatment for *Mycobacterium ulcerans* infection [8]. We showed that adding clarithromycin to rifampicin resulted in a 60% non-significant, but possibly relevant, increase in plasma rifampicin concentration. In addition, we found that the exposure to the drug metabolite 14-hydroxyclarithromycin [9] was significantly higher than that to clarithromycin. This is probably an effect of rifampicin co-medication, inducing the clarithromycin metabolism in the liver [10-12]. The minimal inhibitory concentration (MIC) of 14-hydroxyclarithromycin for *Mycobacterium ulcerans* was studied by Deepak Almeida at the mycobacteriology laboratory of Professor Grosset at the Johns' Hopkins, Baltimore. The MIC appeared too high to expect any additional antimycobacterial effect on *Mycobacterium ulcerans*. The median time above MIC for the Ghanaian *Mycobacterium ulcerans* strain [13] (0.25 mg/l) was about 4 hours per 24 hours. In all eight patients, blood concentrations remained above MIC for at least some period of time. However, for the Malaysian *Mycobacterium ulcerans* strain [14], two patients did never reach the MIC (0.5 mg/l). Neo-macrolide antibiotics, like clarithromycin, have different pharmacokinetics and pharmacodynamics than other antibiotic classes [15]. This class of antimicrobial agents is characterized by a combination of low serum concentrations and high concentrations in phagocytes, while there is debate about actual soft tissue concentrations [16-18]. As *Mycobacterium ulcerans* has an intracellular stage [19-21], clarithromycin might particularly be effective during this phase of infection [22]. Nevertheless, we concluded that, to ensure higher levels of exposure and time above MIC, a dose of 7.5 mg/kg should be given twice daily – or an extended release formulation once daily –, as the dose which was used presently appeared to be well-tolerated. In this way, the chance of inadvertent mono-therapy as a result of drug-drug interaction can be brought to minimum, reducing the chance of inducing drug resistance for rifampicin as well as for clarithromycin.

In chapter 4 we supported our hypothesis on paradoxical reactions accompanying effective treatment for *Mycobacterium ulcerans* infection by describing three observational phenomena in the evolution of disease after start of therapy: (i) transient increases in lesion size; (ii) ulceration of non-ulcerative lesions; and (iii) development of new lesions [23]. The three response patterns were observed during therapy, as well as after the 8 week antimicrobial therapy had ended. For the lesion size analysis 134 Buruli ulcer patients were included; these were participants of the randomized trial described in chapter 2. To be able to show response patterns during effective antimicrobial treatment, included for the lesion size analysis were

participants that healed on treatment. Participants that failed on treatment, were HIV co-infected, or had skin grafting were excluded. Peak 'paradoxical response' occurred at week 8, when over 30% of patients showed an increase in lesion size as compared to the previous (week 6) assessment. To strengthen the analysis, we performed a sensitivity analysis by varying the definitions of paradoxical response, e.g. two consecutive increases or an increase after a decrease in surface area, which, likewise, confirmed the presence of the phenomenon we describe as paradoxical response. A total of 90 participants had non-ulcerative lesions at time of inclusion; 75 (83%) of these lesions ulcerated after start of treatment. Nine participants developed new lesions during or after treatment. All lesions subsequently healed. We concluded from this analysis that, after start of antimicrobial treatment for Buruli ulcer, new or progressive ulceration is common before healing sets in. Moreover, the chapter emphasizes that this paradoxical response should not be misinterpreted as failure to respond on treatment [24,25].

In chapter 5 we showed that early detection of aminoglycoside-induced ototoxicity in a field setting by means of a portable audiometer – with no soundproof audiometry facilities – is limited (manuscript submitted). Though aminoglycoside-induced ototoxicity affects the higher frequencies first [26,27], intra- and inter-observer measurements were less reliable at 6 and 8 kHz. However, in the normal hearing range (0.25 to 4 kHz), the intra- and inter-observer reliability of the portable audiometer was satisfactory. The study showed that a portable audiometer can be well operated in the field by different observers with minimal training, which is useful in general daily practice. Levels of hearing impairment might be overestimated as a result of ambient noise, but changes over time can be reliably detected.

In chapter 6 we describe a patient-control analysis in which significantly lower vitamin D blood concentrations were found in the 179 Buruli ulcer patients as compared to the 180 matched control subjects (65.9 vs 73.0 nmol/L; $p<0.001$) (manuscript submitted). Possible explanations for the lower vitamin D serum concentrations are increased consumption of vitamin D by cells of the immune system at the site of infection [28-30], or less likely, decreased exposure to sunlight resulting from covered skin or inability to participate in farming and other out-door activities, due to *Mycobacterium ulcerans* disease. This new finding could be important in finding novel ways to improve disease management.
We were unable to confirm the earlier established association of SLC11A1 gene polymorphisms with susceptibility for Buruli ulcer disease [31]. The sample size was

similar to the previous study, but the population presently studied had a different ethnic background (i.e., other tribal origins), which could be a possible explanation for the conflicting results. In our patient population, we were unable to detect polymorphisms in the VDR gene or in the MBL gene that were associated with developing Buruli ulcer disease.

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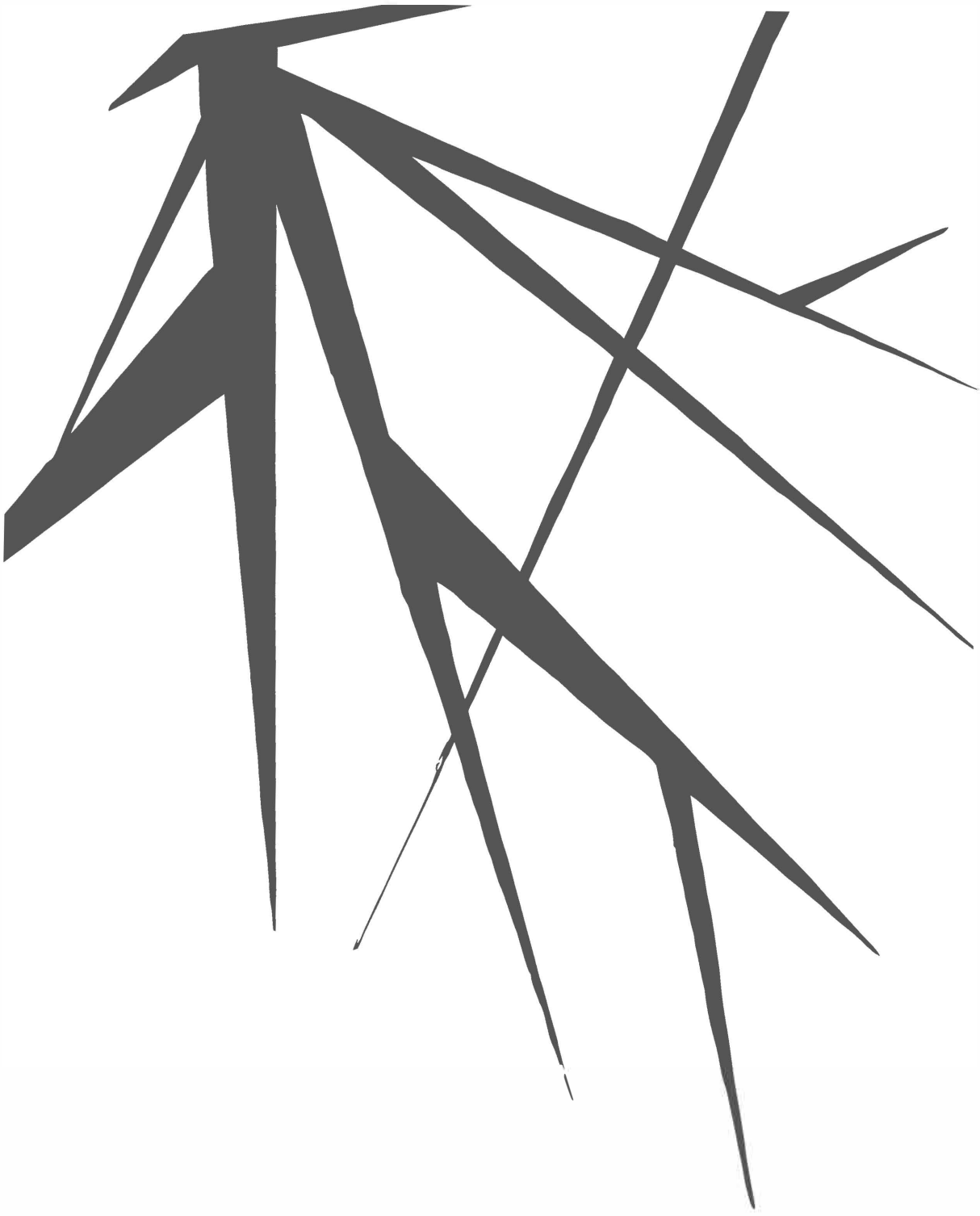

CHAPTER 8

General discussion and future perspectives

With the study described in chapter 2 [1], we intended to make the first step towards developing a fully oral regimen for the treatment of *Mycobacterium ulcerans* infection. Although a switch to oral drugs after four weeks of standard streptomycin injection-based treatment is advantageous in reducing potential additional infectious risks associated with the injections, as well as in reducing logistic challenges incurred by injection therapy in rural Africa [2-5], a fully oral regimen would be the preferred treatment [6-10]. The findings of the present trial led to the preparation of a new trial, addressing the question whether a fully oral treatment is non-inferior to the standard streptomycin and rifampicin treatment.

Apart from finding new and better antimycobacterial treatment combinations, the minimal duration of antimicrobial therapy to cure infection should be another focus of investigation. We hypothesize that antimicrobials shut down mycolactone synthesis so as to allow the immune system to recover and initiate an immune response adequate to fight *Mycobacterium ulcerans* [11-17]. Once this adaptive immune response is established, infection might be controlled without additional antimicrobial treatment. Support for this hypothesis comes from our observation of ultimately healed lesions in which living bacteria were found after the 8 week antimicrobial treatment had been discontinued [1]. Besides, an unknown but considerable proportion of individuals is able to spontaneously clear pre-clinical or clinical infection, without active treatment, which supports the major importance of an adequate immune response in defense for this particular mycobacterium [18-26]. The virulence of *Mycobacterium ulcerans* depends on its formation of mycolactone [27-31]. Arresting the mycolactone synthesis machinery by interfering with (post-) translation of the mycolactone synthesis genes on the plasmid and the cellular genome, the immune system might control the infection. In this view, even single-drug treatment would possibly be adequate in treating *Mycobacterium ulcerans* infection. No resistant *Mycobacterium ulcerans* strains have been cultured from human tissues to date. Indeed, the risk of spreading resistant mycobacteria is much smaller in *Mycobacterium ulcerans* infection than in e.g. tuberculosis, in which, unlike Buruli ulcer, humans are the reservoir of the infectious agent. In fact, unpublished information shows that Buruli ulcer has been treated with rifampicin alone in many endemic areas in the seventies and eighties of the twentieth century [10]. WHO discourages the use of mono-therapy for *Mycobacterium ulcerans* infection. One might argue that even a more wide-spread policy of mono-therapy of an assumedly environmentally acquired infection would probably not be associated with an appreciable risk of drug resistance. The most important concern is however that such treatment

might be associated with recurrence or persistent infection, especially in immune-compromised hosts.
Further, aspects of local treatment, like type of dressing materials [32], frequency of dressing changes, the role of skin grafting as well as the role of limited surgical excision of necrotic tissue in larger lesions [33] should be studied, intending to fasten the slow pace of healing shown in the present study. Topical use of autologous blood products like platelet gel or fibrin sealant [34-38] could be suitable to fasten wound healing as preparation is simple and therefore applicable in resource poor circumstances.
The role of mycolactone, which could have an important role in the slow pace of wound healing, should be another focus of research. The kinetics of mycolactone in tissue and blood should be studied [39]. Ways to halt or decrease mycolactone production, to increase or stimulate its tissue washout, or products that have the ability to degrade the molecule should be developed and tested in vitro.
Apart from its antibacterial effects, clarithromycin is known to have short- and long-term anti-inflammatory effects [40-43]. Immunomodulation might result in faster healing, fewer or less intense paradoxical reactions, or other anti-inflammatory benefits. In the present study we were not able to find support for this, but when clarithromycin is given from start of treatment this positive impact on inflammation might become obvious.

Strikingly, the majority of our patient population presented with lesions on the right side of the body, an observation that has been reported previously in Ghana as well [44,45]. This confirmed finding could be an important additional clue in unraveling the mode of disease transmission. Treatment of *Mycobacterium ulcerans* infection might become superfluous if transmission could be prevented. Despite large and intense research efforts, neither the reservoir nor the transmission to humans has been elucidated [46]. The study of reservoir and transmission is complicated by the fact that transmission can vary over time [47-49]. Possibly several different modes of transmission occur in different endemic foci [46]. For the first years to come, the main focus of research should be improving patient treatment. In the absence of adequate prevention, early diagnosis and prompt and adequate treatment should prevent large lesions with subsequent important sequelae and functional limitations [50-52].

Future clinical antibiotic studies for Buruli ulcer treatment should be accompanied by more extensive pharmacokinetic and -dynamic studies, to confirm drug intake,

and to confirm adequate drug concentrations in blood and especially in affected tissues. Limited tissue sampling techniques combined with minimally invasive finger prick blood specimens air-dried on filter paper which simplify transport conditions could be helpful to study drug-exposure in a larger number of patients [53], and mycobacterial drug resistance testing should be carried out when possible [54,55].

Cost-benefit analyses could strengthen the results of antimicrobial treatment for Buruli ulcer; more attention to this important factor should be given in future trials, as well as to the prevention of disability. Disabilities are obviously less when surgery is brought to a minimum, but without surgery, lesions on or close to joints are still prone to heal with functional limitations [56]. Providing appropriate tools and specialized personnel in the field of prevention of disabilities should be an integrated part of Buruli ulcer treatment, and importantly in future clinical trials money should be budgeted for this, which should improve quality of life for affected individuals.

In the analysis of paradoxical worsening of disease under antibiotic treatment, we could not link the wound surface area measurements with biomarkers or histopathology to confirm our observations with biological phenomena that might explain our findings. Since we first reported our observations of paradoxical responses after start of antibiotic treatment at the WHO meeting on Buruli ulcer in Geneva 2009, case reports that combined clinical and histopathological findings on this phenomenon were subsequently reported [14,16]. To study the phenomenon in a large clinical trial, biomarkers obtained in repeated blood samples in this respect is a more patient friendly way than obtaining consecutive multiple histo-pathological specimens. Future studies should focus on exploration of biomarkers to differentiate paradoxical responses from treatment failure, as a paradoxical response is a reason to continue antibiotic treatment or wait-and-see, while treatment failure would be a reason to switch antibiotic treatment, or to proceed to more costly and potentially disfiguring surgical interventions.

No studies have addressed the question how paradoxical reactions should be prevented or managed. The immunosuppressive drug prednisone has been used for treating paradoxical, immune-mediated responses in tuberculosis, but no association was found between the use of steroids and the duration of the reaction [57]. Steroids might be efficacious especially when the central nervous system is involved; they might reduce symptoms by controlling disease associated edema [58]. The 'immune reconstitution inflammatory syndrome' (IRIS) is a paradoxical response

following immune recovery in the context of starting antiretroviral therapy for HIV infection [59,60]. IRIS is associated with inflammation in response to an infectious agent, sometimes to dead bacillary antigens, and may present as the unmasking of an initially subclinical co-infection, or as a deterioration of a co-infection under treatment [61,62]. In paradoxical tuberculosis-associated IRIS, the use of steroids has been addressed in a randomized clinical trial [63]. Prednisone provided benefit as it reduced the need for hospitalization and therapeutic procedures, hastened improvements in symptoms and quality of life, despite a higher risk of nonsevere opportunistic infections. Case reports and series describe NSAIDs, thalidomide and leukotriene receptor antagonists as potentially beneficial; and TNF-α inhibitors, chemokine receptor antagonists, vitamin D and statins have been suggested to prevent IRIS or its complications [60]. These agents might have a possible beneficial role in treating or preventing paradoxical responses in *Mycobacterium ulcerans* infection as well.

To explore possible genetic associations with Buruli ulcer, response to treatment–(time to) healing, paradoxical reaction–, pharmaco-genetics as well as pharmacokinetics and -dynamics, future studies should enroll larger patient populations. Whole Genome Analysis could be a next step to gain more knowledge on these issues, to be able to use this knowledge to improve treatment strategies. An international bio bank could be set up, which provides the opportunity to strengthen collaboration in Buruli ulcer research and to merge small databases and scattered patient information.

The interesting finding of serum vitamin D concentrations, which were lower in untreated Buruli ulcer patients as compared to healthy controls [64] should be followed by a confirmation study. This study could also incorporate vitamin D measurements after treatment has finished and at the moment patients are healed, to differentiate pre-existing differences in vitamin D status from vitamin D consumption by cells of the immune system or a decrease in sunlight exposure resulting from covered skin, stigma, or the impossibility to work outside. Furthermore, our proposed possible role of cathelicidin [65], a peptide that is related to vitamin D dependent antimicrobial effects should be investigated, which might give clues to fight Buruli ulcer disease in different ways. Finally, supplementing vitamin D might be worth studying, as has been tried in tuberculosis [66,67].

In conclusion, the randomized trial we performed has clearly answered the question whether patients with *Mycobacterium ulcerans* infection should have antimicrobial

treatment:the evidence base is sufficiently strong for a high grade of recommendation [7].All-oral schemes should be developed, ways to fasten healing should be provided and the newly introduced phenomenon of paradoxical response to treatment should be studied and explored with the intention to provide definitions, criteria and treatment recommendations to optimize treatment for Buruli ulcer.

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Nederlandse samenvatting

Buruli ulcus

Buruli ulcus (Buruli zweer of afgekort BU) is een tropische, zwerende huidinfectie veroorzaakt door *Mycobacterium ulcerans*. Toen in de jaren zestig uit Oeganda, onder andere uit het Buruli-district, vele meldingen kwamen over het vóórkomen van de infectie, raakte de naam 'Buruli ulcus' in zwang. Na tuberculose en lepra, die net als BU door mycobacteriën, een speciale groep micro-organismen, worden veroorzaakt, is BU de derde belangrijkste mycobacteriële infectieziekte bij mensen met een normale afweer. De bacterie – *M. ulcerans* – kan in het laboratorium gekweekt worden en groeit het beste bij temperaturen rond 32 °C. De bacterie heeft als unieke eigenschap dat het in staat is om een giftige stof – mycolacton – te maken; deze stof is de oorzaak van de uitgebreide weefselschade zoals die bij patiënten, en ook bij proefdieren optreedt.

BU komt op dit moment met name voor in West-Afrika. Het merendeel van de patiënten woont in arme, afgelegen gebieden, in een moerassige of vochtige omgeving. De infectie begint als een subcutane nodus (onderhuidse bobbel), een plaque (plaatachtige verharding van de huid) of oedeem (zwelling gepaard met vochtuittreding in weefsels), die samen aangeduid worden als de niet-ulceratieve lesies. Op een gegeven moment breekt de huid open en ontstaat er een zweer met ondermijnde rand en centraal veel dood weefsel, de ulceratieve lesie (zie plaatjes bij hoofdstuk 1). De infectie is pijnloos en wordt vooral gezien op de armen en benen van kinderen. Het is onbekend hoe de infectie wordt opgelopen. De Wereldgezondheidsorganisatie (WHO) schaart BU – samen met 18 andere tropische aandoeningen – onder de "verwaarloosde tropische ziektes". In hun *Plan to combat Neglected Tropical Diseases (2008-2015)* stelt de WHO dat er geen bevredigende mogelijkheden zijn om de infectie te bestrijden. Dit proefschrift richt zich op de effectiviteit van antibiotica voor de behandeling van BU.

Moet Buruli ulcus behandeld worden met antibiotica?

Hoofdstuk 1 geeft een inleiding in de geschiedenis van de behandeling van BU. Bij tuberculose en lepra bestaat de belangrijkste behandeling uit een combinatie van ten minste twee anti-mycobacteriële middelen. Bij BU leken deze antibiotica niet te werken: er was tijdens de behandeling geen duidelijk effect zichtbaar en soms namen de afwijkingen alleen maar toe in grootte en ernst. Lang heeft men daarom gemeend dat een operatieve verwijdering van aangedaan weefsel de enige goede kans op genezing bood. Hoewel *M. ulcerans*, de bacterie die BU veroorzaakt, bij testen in het laboratorium gevoelig bleek voor verschillende antibiotica, en deze antibiotica ook effectief waren in een diermodel waarbij *M. ulcerans* werd ingespoten

in voetkussens van muizen, waren de resultaten van behandeling met antibiotica in de praktijk vaak teleurstellend. De principiële vraag of antibiotica in staat zijn *M. ulcerans* bij de mens uit te schakelen, moest eerst beantwoord worden. Door een studiegroep van de WHO werd daarom een onderzoek opgezet en uitgevoerd waarbij uiteindelijk 21 patiënten met een onderhuidse nodus door *M. ulcerans*, één van de niet-ulcererende vormen van BU, enkele weken werden behandeld met een combinatie van twee antibiotica. De keuze viel op twee middelen – streptomycine (S), een middel dat alleen als injectie gegeven kan worden, en rifampicine (R), wat als tablet kan worden toegediend. Op basis van eerdere experimenten met meerdere antibiotica - zowel in kweekproeven als in diermodellen, was de combinatie SR als de meest krachtige naar voren gekomen. Na de antibiotische behandeling die steeds varieerde in tijdsduur, werd de lesie operatief verwijderd, en het weefsel gekweekt. Bij de patiënten die twee weken waren behandeld met SR werden nog levende bacteriën aangetoond in het verwijderde weefsel, maar bij geen van de patiënten die vier, acht of twaalf weken waren behandeld werden nog levende bacteriën geïsoleerd. Op basis van deze resultaten die tijdens de WHO Buruli ulcus bijeenkomst in 2004 werden gepresenteerd en later ook gepubliceerd, ontstond een omslag in het denken over de rol van antibiotica bij de behandeling van BU. Antibiotica werden steeds meer voorgeschreven – zeker daar waar geen mogelijkheden voor chirurgische behandeling voorhanden waren. Het effect van antibiotische behandeling bij de meest bekende presentatie van de ziekte – het ulcus – was echter niet onderzocht in een goed opgezet klinisch (patiënt-gebonden) wetenschappelijk experiment. Ook was onbekend of de niet-ulceratieve lesies bij de patiënten in het beschreven onderzoek inderdaad genezen waren als deze niet chirurgisch verwijderd zouden zijn. Is de dosering en behandelduur die gekozen was voor de patiënten met de nodus vorm van BU afdoende voor alle vormen van BU? Hoe groot is de kans op genezing als geen operatie wordt uitgevoerd naast antibiotische behandeling? Is er kans op antibiotica resistentie, bijvoorbeeld door te lage dosering met te lage weefselconcentratie van antibiotica, of kan de behandeling falen door een te korte behandelduur? Hoe groot is de kans op bijwerkingen en toxiciteit (schade) door antibiotische behandeling? Is de genezing blijvend of komt de ziekte weer terug, bijvoorbeeld een jaar na staken van de behandeling, zoals vaak het geval is bij chirurgische behandeling? Acht weken dagelijkse injecties met streptomycine is vooral voor kinderen maar ook voor volwassenen een probleem. Op het Afrikaanse platteland waar BU het meest voorkomt is het moeilijk om injecties op steriele wijze te geven; gezondheidsposten zijn soms nog ver verwijderd van de plaats waar de patiënten wonen; en de overdracht van HIV door ongelukken met injectiespuiten is

in Afrika ten zuiden van de Sahara aanzienlijk. Is het mogelijk om het aantal injecties te beperken en althans een deel van de kuur met andere antibiotica te behandelen? Hoewel al deze vragen nog onbeantwoord waren was de druk om antibiotica in te voeren zo groot dat de WHO in 2004 in een voorlopige richtlijn adviseerde om BU voortaan te behandelen met de SR combinatie. De behandelduur evenals de beslissing om aanvullend te opereren werd aan het klinisch oordeel overgelaten: SR 8 tot 12 weken werd als standaard therapie geadviseerd, met eventueel chirurgie, ook al was duidelijk dat er aanvullend onderzoek nodig was om de nog vele openstaande vragen te beantwoorden. Hoofdstuk 1 beschrijft, in relatie tot deze vragen, de opzet van het proefschrift met de onderzoeksvragen die in de volgende hoofdstukken worden getoetst.

Wat is de werkzaamheid van de 'standaard' (door de WHO geadviseerde) behandeling van Buruli ulcus met 8 weken streptomycine + rifampicine, zonder uitgebreide chirurgie? Is de behandeling ook effectief wanneer het injecteerbare antibioticum (streptomycine) na 4 weken wordt vervangen door tabletten (claritromycine)?

In *hoofdstuk 2* wordt de werkzaamheid van antibiotica voor de behandeling van BU onomstotelijk aangetoond. De 'standaard behandeling' streptomycine en rifampicine gedurende 8 weken (8SR) werd vergeleken met een schema van rifampicine gedurende 8 weken en streptomycine gedurende maar 4 weken, waarna de behandeling werd voortgezet met claritromycine (C) gedurende 4 weken (4SR/4CR). Dit laatste schema heeft als voordeel dat de injecties na 4 weken worden omgezet in tabletten. 151 deelnemers met een vroege (niet langer dan 6 maanden bestaande), beperkte (niet groter dan 10 cm in diameter) vorm van BU werden, nadat zij hiervoor toestemming hadden gegeven, willekeurig verdeeld over de twee behandelschema's. De loting voor één van de twee behandelschema's werd uitgevoerd met behulp van een computerprogramma in Nederland, vervolgens werd de toewijzing overgezonden; de communicatie verliep via SMS berichten tussen Nederland en Ghana. De steekproefgrootte was zodanig gekozen dat wanneer tenminste 148 deelnemers tot het einde van het onderzoek – 52 weken na start van behandeling – gevolgd zouden worden, een voldoende betrouwbaar antwoord gegeven kon worden op de onderzoeksvraag: hoe vaak treedt genezing op zonder terugkeer van ziekte? Dit primaire eindpunt werd vergeleken tussen de deelnemers van de twee verschillende behandelschema's. Vier van de 151 deelnemers konden om verschillende redenen niet worden gevolgd tot week 52, maar op het moment dat zij voor het laatst gecontroleerd werden was hun lesie genezen; zij werden daarom ook meegenomen

in de analyse. In totaal was er bij 11 van de 151 deelnemers sprake van 'therapiefalen': de lesie van vijf deelnemers verslechterde in die mate dat behandelteams besloten tot uitgebreide chirurgie, en de lesie van zes deelnemers was nog niet genezen op week 52. Dit resulteerde in een statistisch gezien niet verschillende behandeleffectiviteit van 96% (73/76) in de 8SR groep en van 91% (68/75) in de 4SR/4CR groep. In geen van beide groepen trad een recidief op van het ziektebeeld. Vijf deelnemers werden behandeld met behulp van een huidtransplantatie vanwege de grootte van het huiddefect. Er werden weinig bijwerkingen gemeld; drie deelnemers klaagden over duizeligheid (gevolg van streptomycine); drie meldden buikklachten; één ontwikkelde een injectie abces. Twee deelnemers ontwikkelden een abces in de buurt van de oorspronkelijke lesie. Uit deze studie kan geconcludeerd worden dat antibiotische behandeling gedurende 8 weken uitermate effectief is voor BU patiënten met een vroege, beperkte infectie, en dat injectietherapie verkort kan worden naar 4 weken door de patiënten verder met tabletten te behandelen.

Beïnvloeden rifampicine en claritromycine elkaars werkzaamheid en wat heeft dat voor consequenties voor de behandeling van Buruli ulcus?

In *hoofdstuk 3* wordt de complexe wisselwerking tussen twee van de in hoofdstuk 2 gebruikte antibiotica, rifampicine en claritromycine – wanneer deze middelen tegelijkertijd worden toegediend – bestudeerd. Rifampicine kan de lever aanzetten tot een versnelde afbraak van bepaalde stoffen, waaronder claritromycine. Dit kan leiden tot een lagere concentratie van claritromycine in het bloed, met als eventueel ongewenst effect een lagere werkzaamheid en resistentievorming, dat wil zeggen het ongevoelig worden van de bacterie voor het antibioticum door een onvoldoende krachtige behandeling. Anderzijds zorgt claritromycine voor een vertraagde afbraak van bepaalde stoffen in de lever, waaronder rifampicine. Dit kan juist resulteren in een verhoogde concentratie van rifampicine in het bloed, waardoor er sneller bijwerkingen zouden kunnen ontstaan. Voor dit type onderzoek waarbij het verloop van bloedconcentraties in de tijd worden vergeleken, worden meestal kleine aantallen deelnemers onderzocht; 13 deelnemers uit de studie beschreven in het vorige hoofdstuk namen deel aan dit farmacologische onderzoek. Gedurende één behandeldag werd frequent bloed afgenomen om het verloop van de concentraties van de antibiotica in de tijd te kunnen bepalen. De inname van claritromycine in de onderzochte patiënten resulteerde in een 60% hogere bloed concentratie van rifampicine. In deze kleine groep deelnemers was het verschil niet significant, maar mogelijk wel van belang. Verder bleek dat bij de meeste deelnemers gedurende tenminste enige tijd van de dag concentraties van claritromycine in

het bloed werden bereikt die hoog genoeg waren om de groei van *Mycobacterium ulcerans* tegen te gaan. De concentratie van het afbraakproduct van claritromycine, 14-hydroxy-claritromycine, bleek veel hoger dan van claritromycine zelf. Hierop werd samenwerking tot stand gebracht met het laboratorium van Professor Grosset in het Johns Hopkins ziekenhuis in Baltimore. Door de groep van Grosset werd onderzocht of deze afbraakstof – die naast het antibioticum zelf bij andere bacteriën vaak een goed antibiotisch effect heeft – ook invloed heeft op de groei van *Mycobacterium ulcerans*. De antibiotische werking van 14-hydroxy-claritromycine op *M. ulcerans* bleek zwak en niet bijdragend.
Claritromycine is onderdeel van een groep antibiotica genaamd neo-macroliden, die hun antibiotische werking uitoefenen op bacteriën door de eiwit aanmaak in de bacterie te remmen. Neo-macroliden hebben een opvallend lage concentratie in het bloed, maar juist een hoge concentratie in cellen. Met name fagocyten, dit zijn afweercellen die een rol spelen in het 'opeten' van bacteriën, kunnen hoge concentraties bevatten. Omdat *M. ulcerans* in de eerste fase van de infectie in de cel zit, is mogelijk tijdens deze fase de activiteit van claritromycine tegen de bacterie het sterkst. Desondanks concluderen wij aan het einde van het hoofdstuk op basis van bovenstaande resultaten dat, om voldoende werkzaamheid te garanderen en om resistentievorming te voorkomen, claritromycine in de toekomst liefst hoger gedoseerd moet worden wanneer gecombineerd met rifampicine voor de behandeling van BU, mede omdat met het gebruikte doseringsschema het middel goed werd verdragen.

Waarom heeft men zo lang gedacht dat antibiotica niet werkzaam zijn tegen Buruli ulcus? Is er bewijs voor een met de antibiotische behandeling gepaard gaande paradoxale reactie?

In *hoofdstuk 4* onderzochten en beschreven wij het fenomeen paradoxale reactie bij de behandeling van Buruli ulcus. Een paradoxale reactie wil zeggen dat er tijdens de behandeling een klinische verslechtering van de ziekte optreedt, terwijl de bacteriën die de infectie veroorzaken juist goed onderdrukt worden, en waarbij uiteindelijk de behandeling effectief blijkt te zijn. Dit fenomeen is bekend bij tuberculose (vooral lymfkliertuberculose) en bij lepra. Bij BU werden er enkele gevallen beschreven nadat wij hier in 2009 in een mondelinge presentatie over hadden gerapporteerd. Wij hadden tijdens de uitvoering van het onderzoek beschreven in hoofdstuk 2 opgemerkt, dat het regelmatig leek alsof (i) wondoppervlaktes groter werden voor er genezing optrad, (ii) lesies met een intacte huid eerst openbraken, voordat er genezing optrad, en (iii) er bij patiënten tijdens of na de behandeling nieuwe lesies

ontstonden, die genazen zonder iets in de behandeling aan te passen.
Voor dit onderzoek wilden wij patiënten met een normale afweer onderzoeken, die volgens de tevoren vastgestelde criteria zoals beschreven in hoofdstuk 2 waren genezen. Deelnemers die met HIV geïnfecteerd waren, en ook deelnemers die met een huidtransplantatie of andere operatie waren behandeld werden uitgesloten; de gegevens van 134 deelnemers werden geanalyseerd. Tijdens elke controle werd het wondoppervlak van de lesie van elke deelnemer bepaald – dit door te kijken naar de afwijkingen van de huid, maar ook door de huid ronddom te betasten – waarbij de afwijking die in de huid werd gevoeld ook werd gemarkeerd op de huid. Met behulp van een doorzichtige sheet werd dan de grootte van de lesie vastgelegd. Deze sheet werd gedesinfecteerd en bewaard, om later ingescand te worden. Met behulp van een grafisch programma werd het wondoppervlak met de computer berekend. Per deelnemer werd het verloop van het oppervlak van de lesie bekeken door elke meting te vergelijken met de meting ervoor. Hierbij viel op dat er in week 8 een piek werd gezien in het optreden van een paradoxale respons; meer dan 30% van de deelnemers had in week 8 een toename van het wondoppervlak in vergelijking met week 6. Omdat het onbekend is hoe betrouwbaar eenmalige wondoppervlakte metingen zijn, werden meer analyses verricht, waarbij de definitie van paradoxale reactie werd verscherpt, bijvoorbeeld twee opeenvolgende toenames of een toename na een afname in wondoppervlak; ook als we de definitie aanpasten werd het optreden van paradoxale reacties bevestigd. 90 deelnemers hadden bij aanvang van het onderzoek een niet-ulceratieve lesie. Bij 75 (83%) van deze lesies ontstond alsnog een zweer, soms tijdens en soms na afronden van de antibiotische behandeling, voordat genezing optrad. Negen deelnemers ontwikkelden nieuwe lesies tijdens of na de antibiotische behandeling, die alle ulcereerden, maar zonder aanvullende maatregelen weer genazen. De bovenstaande observaties deden ons concluderen dat na het starten van antibiotische behandeling wegens BU vaak nieuwe lesies ontstaan, of bestaande zweren toenemen in grootte voordat genezing optreedt. Deze opvallende bevinding zou kunnen verklaren waarom de effectiviteit van antibiotica in het verleden is onderschat, en dat verkeerde conclusies werden getrokken uit waarnemingen met onvoldoende duur van follow-up. Deze paradoxale reacties moeten dus niet verward worden met therapiefalen. Dit onderscheid is moeilijk omdat wij therapiefalen eerder hadden aangeduid als toename van lesies tot meer dan 150% van de grootte bij het begin van de behandeling. De discussie over paradoxale reacties is natuurlijk niet alleen van groot praktisch belang, maar is ook belangrijk bij het ontwerpen van toekomstige onderzoeken om BU met antibiotica te behandelen.

Is draagbare audiometrie geschikt voor het aantonen van gehoors-vermindering door het antibioticum streptomycine?

Streptomycine is onderdeel van een groep antibiotica genaamd aminoglycosides. Naast een krachtige bacteriedodende werking hebben deze middelen ook bijwerkingen, waaronder gehoorschade, met name wanneer er hoge doseringen worden gegeven of wanneer er langdurig behandeld wordt. In *hoofdstuk 5* laten wij zien dat het vroegtijdig vinden van door aminoglycoside veroorzaakte gehoorschade door middel van een draagbare audiometer in een ziekenhuis zonder geluidsdichte kamer, moeilijk is. De betrouwbaarheid van de draagbare audiometer werd bepaald door deze eerst te vergelijken met de apparatuur van de geluidsdichte kamer in het universiteitsziekenhuis van Kumasi, en daarna door dubbelmetingen met maximaal enkele uren tussenpoos bij verschillende vrijwilligers in de ziekenhuizen waar het onderzoek beschreven in hoofdstuk 2 werd verricht. Hier was geen geluidsdichte kamer en het niveau van achtergrondgeruis was hoog met een geluidssterkte van ongeveer 50 dB. In de 0,5 tot 4 kHz frequenties (dit zijn de toonhoogtes of frequenties waarin de normale spraak zich bevindt) bevond de tweede meting zich in meer dan 95% procent van de gevallen binnen de algemeen geaccepteerde bandbreedte van ±10 dB ten opzichte van de eerste meting. In de 6 en de 8 kHz was de betrouwbaarheid van de test minder goed. Omdat hoorschade als gevolg van aminoglycosides meestal begint bij de hogere tonen, dat wil zeggen in de frequenties die boven het normale spraakgebied liggen, kan het vroeg aantonen van gehoorsvermindering niet met voldoende betrouwbaarheid worden vastgesteld met behulp van de gebruikte apparatuur in de beschreven setting. In de frequenties van het normale spraakgebied is de betrouwbaarheid van de draagbare audiometer acceptabel voor het aantonen van veranderingen van gehoor in de tijd, alhoewel absolute gehoorsvermindering overschat kan worden door achtergrondgeluid. De studie liet overigens ook zien dat de draagbare audiometer goed bediend kan worden door mensen na minimale training, wat bruikbaar is in de dagelijkse praktijk.

Speelt vitamine D een rol in de afweerreactie tegen *Mycobacterium ulcerans*, de verwekker van Buruli ulcus? Spelen normaal voorkomende variaties in genen een rol in de vatbaarheid voor het krijgen van Buruli ulcus?

In *hoofdstuk 6* werden de BU patiënten uit hoofdstuk 2 vergeleken met gezonde controle personen die zo werden gekozen dat ze zo goed mogelijk vergelijkbaar ('gematched') waren met de patiënten. De controle personen werden geselecteerd op grond van het geslacht, de leeftijd en de stam van waaruit de patiënt afkomstig was.

Bij tuberculose en lepra is eerder gebleken dat vitamine D, dat in voeding voorkomt, maar dat vooral onder invloed van zonlicht in de huid wordt aangemaakt, belangrijk is voor beschermende immuunreacties. Voor het antibioticatijdperk was zonlicht een geaccepteerde vorm van behandelen van tuberculose, en inname van extra vitamine D kan in bepaalde gevallen de behandeling van tuberculose gunstig beïnvloeden. In het bloed van de patiënten en de controlepersonen werd het vitamine D gehalte bepaald: deze bleek bij de BU patiënten lager dan bij de controle personen, waarbij het verschil overigens niet groot was (65.9 nmol/L vs 73.0 nmol/L; $p<0.001$). Wij speculeren over mogelijke verklaringen voor deze bevinding, waarbij het kan zijn dat ook bij BU patiënten, net als bij tuberculose en lepra, afweercellen ter plekke van de infectie vitamine D gebruiken om goed te kunnen functioneren. Minder waarschijnlijk is dat een afgenomen aanmaak van vitamine D door een verminderde blootstelling aan zonlicht, vanwege het bedekken van de huid door stigma of het niet kunnen uitvoeren van werkzaamheden op het land door functionele beperkingen, hiervan de oorzaak is. De bevinding zou van waarde kunnen zijn om de ziekte in de toekomst beter te kunnen begrijpen en te behandelen.
Niet alle personen die in aanraking komen met *Mycobacterium ulcerans* ontwikkelen BU. In eerder onderzoek werd aangetoond dat bepaalde natuurlijk voorkomende varianten (polymorfismen) in een bepaald gen (SLC11A1) de kans op het krijgen van BU in zeer sterke mate verhoogde; eerder was dit effect ook al beschreven bij lepra en tuberculose. Dit gen is betrokken bij transport van zware metalen en heeft vermoedelijk een functie bij de afweer tegen bacteriën in de macrofaag (de afweercellen die bacteriën 'opeten'). Wij besloten in het bloed van onze patiënten en controles hetzelfde gen te onderzoeken, waarbij de uitslag van het eerdere onderzoek niet kon worden bevestigd. Dit is mogelijk te verklaren door verschillen in genetische afkomst van onze patiënten in vergelijking met die van het eerder uitgevoerde onderzoek. Naast dit gen werden twee andere genen onderzocht, namelijk het vitamine D receptor gen, dat de gevoeligheid voor vitamine D mede bepaalt; en het MBL gen, omdat variaties in dit gen de vatbaarheid voor krijgen van tuberculose en lepra, de twee andere belangrijke mycobacteriële infectieziekten, kan beïnvloeden. Net zoals het SLC11A1 gen bleken het MBL en het vitamine D receptor gen in onze patiënten en controles niet te verschillen, althans niet voor wat betreft de onderzochte variaties.

Samenvatting

Hoofdstuk 7 geeft een Engelstalige samenvatting van het proefschrift.

Discussie en toekomstperspectieven

Hoofdstuk 8 geeft een afsluitende discussie met ideeën voor verder onderzoek, als mogelijk vervolg op de in dit proefschrift beschreven studies. Een belangrijke vervolgstap zou zijn het verder ontwikkelen van een volledig orale antibiotische behandeling van BU (met tabletten, zonder injecties). De voorbereidingen voor een studie die een volledig oraal schema zal vergelijken met de standaard 8SR injectietherapie zijn in volle gang. Onze studie heeft slechts een tevoren vastgestelde behandelduur voor BU geëvalueerd. Of de 8 weken durende antibiotische behandeling eventueel verkort zou kunnen worden of in bepaalde gevallen juist langer zou moeten duren is nog onduidelijk. Ook is nog behoefte aan onderzoek naar verbetering van de lokale wondbehandeling; wondverzorging met moderne verbandmiddelen lijken op het eerste gezicht kostbaar, maar als het aantal verbandwisselingen kleiner is zou dit misschien toch geld en mankracht besparen, en comfort voor patiënten bieden. Ook zouden bepaalde chirurgische technieken wellicht de genezing van BU nog kunnen versnellen. Ons onderzoek naar paradoxale reacties bleef beperkt tot het beschrijven en analyseren van observaties. Er is onderzoek nodig naar het vinden van de immunologische mechanismen en daaraan gepaard, het vinden van immunologische merkers (tests, bijvoorbeeld in een bepaling in een bloedmonster, die de aanwezigheid van een paradoxale reactie aangeven) voor dit fenomeen. De belangrijke uitdaging is om therapiefalen van een paradoxale reactie te onderscheiden, omdat een paradoxale reactie een uiting is van een in principe adequate behandeling, en therapiefalen juist het tegenovergestelde. Ook weten we niet hoe paradoxale reacties therapeutisch benaderd moeten worden. Bij tuberculose en lepra is het gebruikelijk, om deze reacties te onderdrukken met afweer-onderdrukkende medicatie zoals prednisolon. Moeten paradoxale reacties ook bij BU geremd en behandeld worden met aanvullende medicatie?

Meer informatie over genetische aanleg voor het ontwikkelen van BU, na het in aanraking komen met *M. ulcerans*, zou inzicht kunnen geven in welke personen speciale maatregelen zouden moeten treffen om de ziekte te voorkomen.

De lagere concentratie vitamine D die werd gevonden in bloed van patiënten in vergelijking met gematchte controlepersonen moet door nieuw onderzoek worden bevestigd. De rol van vitamine D bij BU zou ook in laboratoriumexperimenten moeten worden ontrafeld. Ook zou bekeken kunnen worden of het vitamine D gehalte van succesvol behandelde patiënten na verloop van tijd weer stijgt, en wat dit zou kunnen betekenen voor de (aanvullende) behandeling van patiënten.

Concluderend heeft ons onderzoek de vraag beantwoord of BU behandeld moet worden met antibiotica: er is voldoende bewijs om antibiotica te beschouwen als de basis voor de behandeling. Nieuwe antibiotische schema's moeten ontwikkeld worden; de behandelduur bij patiënten met hele kleine of juist hele grote afwijkingen moet eventueel aangepast worden; een snellere genezing kan mogelijk bereikt worden door onderzoek naar verbetering van de wondbehandeling (verbandmiddelen, eventueel met toepassing van op de wond aangebrachte middelen; en misschien bepaalde vormen van chirurgie). Er is beter inzicht nodig in het wezen van een paradoxale reactie, en daaraan gekoppeld, het ontwikkelen van tests om het onderscheid met therapiefalen te maken; en het ontwikkelen van een behandeling van patiënten met zo'n reactie is tenslotte een nog verder verwijderd doel.

Dankwoord

In het Twii bedank je mensen door te zeggen *meda wo ase*, wat letterlijk betekent *ik leg mij aan je voeten*. Ik vind het een prachtige uitdrukking, één die veel respect toont. Ik wil mij dan ook figuurlijk aan jullie voeten leggen. Jullie zijn allemaal in meer of in mindere mate onderdeel geweest van het hele proces: zonder jullie zou dit proefschrift er niet zijn geweest of niet zijn zoals hij nu is.

Allereerst ben ik mijn patiënten veel dank verschuldigd. Jullie stelden vertrouwen in ons en kwamen steeds maar weer voor de geplande controles, zelfs al waren wonden genezen en hadden jullie belangrijkere dingen aan je hoofd dan een drug trial. Ik heb veel respect voor jullie!

Beste Prof. dr. van der Werf, mijn promotor, Tjip. Jouw enthousiasme voor het onderwerp is besmettelijk. Ik ken niemand die zo snel reageert op 'drafts'. Zonder elkaar gesproken te hebben vroeg je mij dit onderzoek te coördineren. Onze verschillende karakters botsten wel eens, wat we misschien niet eens zo vaak doorhadden vanwege de grote letterlijke afstand die vaak tussen ons in zat. Zonder jou was het onderzoeksplan er niet geweest, was het uitgevoerde onderzoek niet van deze kwaliteit geweest en was ook dit proefschrift niet geschreven. Jij benaderde mij soms met vaderlijke strengheid, maar gaf complimenten wanneer je ergens tevreden over was. Heb ik je al bedankt voor het mij over de lijn dwingen? Als jij dat niet had gedaan dan zou ik nu nog steeds zeggen: ik ben ermee bezig! Wat heerlijk dat het nu af is. Mijn grote dank.

Beste Dr. Stienstra, mijn co-promotor, Ymkje. Ik mocht voortborduren op jouw ingezette onderzoek. Ik heb altijd veel bewondering voor je gehad, bedankt dat je mij wilde begeleiden en sturen, dit ondanks je sommige ergernissen over mijn vertragingen. Jij bent erg praktisch en oplossingsgericht, en vaak dacht ik ná met jou gepraat te hebben: oh ja zo kan het ook! Jouw ontzettend veel goede ideeën, tips en adviezen over de methodiek, de tekst en de onderbouwing in de manuscripten maakten mij wel eens moe, maar ik ben er je dankbaar voor. Ze dwongen mij om na te blijven denken.

Dear Kabiru Mohammed Abass, without you we would still not have reached our calculated sample size! Your positivity, enthusiasm, discipline and great talent to motivate people are priceless. It was just great to work with you in Agogo, and I value you and your wife's friendship highly. Dear Wilson Tuah, thanks for your always inexhaustible energy to run the Buruli clinic in Nkawie. Not only one day a week, but always when necessary you gave your time. Your accurate character and laboratory skills were of great help, and made you a very suitable person to help in the research.

Dear Victoria Ahiable (Vic), Mr. Frimpong, Edmund Atakora (Wofa Yaw) and Justice Abotsi: together we've traced, seen and treated lots of patients! I admire every one of you for the time and the effort you have shown in sometimes difficult circumstances. We had great times together. Dear Mr. Dyabaah (head Agogo Community Health Office), Juliana Agyeibei (nurse/midwife health post Ananekrom), Josephine Kyerewaah, Janet Duah-Agyekum and Yaw Gyewonu (theatre nurses Nkawie). Without you the trial would not have succeeded.

Dear Dr.. Peter Awuah and Dr. William Thompson, thank you for welcoming me as a stranger in your hospital, and thank you for your collaboration and advise whenever necessary. Dear Prof. Ohene Adjei, I remember you brought me to Ghana for the first time, when we came from Benin. Thank you for taking care of me, and thank you for your guidance. Dear Dr. Thomas Kruppa, thank you too for having me in KCCR. Dear Nana Yaa Awua-Boateng, Vera Siegmund and Dr. Gisela Bretzel: working in a laboratory can be quite stressful, especially in tropical circumstances and when the time-pressure of a randomized trial comes in! Thank you for your collaboration and the desire to deliver high quality work.
Dear Prof. dr. Geoffrey Kwabla Amedofu, thank you for the very nice collaboration at the ENT department of Komfo-Anokye Teaching Hospital.

Dear Dr. Kingsley Asiedu, thank you for your support concerning our project, and for the very important work you do at the WHO office concerning Buruli ulcer. Dr. Edwin Ampadu, thank you for allowing me to somehow be part of your National Buruli Ulcer Control Program when I was in Ghana. I also thank the other personnel at the National Buruli Ulcer Office in Accra for the collaboration.

Dear Dr. Phillips, Richard. You became not only my advisor and tutor, but also my friend, and I appreciated the times I could spend with your great family. I deeply admire you for your ongoing energy to combine patient care with Buruli ulcer research.

I thank the Data Safety Monitoring Board consisting of Prof. William R Faber, Prof. Paul DR Johnson and Dr. Alan J Knell for their willingness to spend time on evaluating the results and the progress of the trial. Dear Cari Stek, thank you for the monitoring activities you performed for the drug trial. It was very nice to have you there.

I am honored that the reading committee consisted of Prof. dr. B.C. de Jong, Prof. dr. B. Fleischer and Prof. dr. W.R. Faber. Your effort is highly appreciated.

Dear Prof. Fleischer, Bernhard. I thank you for the energy consuming work you did on coordinating the BURULICO project, but I also warmly thank you for the moral support you gave me every now and then.
Thanks also to all other collaborators and work packages in the BURULICO project.

Beste Jan Schouten. Jij bent van onschatbare waarde geweest voor belangrijke statistiek in dit proefschrift. Ik vond het altijd weer leuk om met jou hiernaar te kijken. Dank je voor je hulp, zelfs na je pensionering, bij jou thuis.
Beste Jan Breeuwsma, de financiën doen zal nooit mijn favoriete bezigheid zijn, al houd ik er wel van om alles goed op een rijtje te hebben. Dat was in Ghana lastig met al die duizenden bonnetjes en de niet te voorspellen uitgaven. Bedankt voor je structurering en begeleiding.

Beste Dr. Eveline van der Veer, ik wil je bedanken voor je begeleiding betreffende de vitamine D materie. Na lange radiostiltes reageerde je zo enthousiast dat dat mij weer energie gaf. Ook Jan en Karin Koerts wil ik bedanken voor de snelle vitamine D bepalingen.
Beste Prof. Dr. Pieter Dijkstra, dank je voor je altijd positieve input bij dat ene voor mij lastige artikel waar jij bij betrokken werd. Jouw advies om mijn eigen keuzes te maken omdat je niet iedereen kan behagen had ik nodig. Beste Prof. Hero Wit, dank u voor de introductie in de voor mij totaal onbekende audiometrie.
Beste Pieter van der Vlies en Cleo van Diemen, nog steeds is genetica en de daarbij behorende statistiek voor mij lastige materie. Het was leuk om met jullie hulp daaraan gesnuffeld te hebben.
Beste Johan Bijzet, bedankt voor je zorg voor mijn monsters. Ik hoop dat wij ooit een eigen vriezer zullen krijgen...
Beste Prof. dr. Bert Niesters en Rob Faber, bedankt voor jullie hulp door middel van virologie bepalingen, tussen alle verbouwingen door.
Beste Marieke de Waard en Janneke Huizinga. Ik ben jullie dankbaar voor het –in jullie vrije tijd– geblindeerd beoordelen van alle Buruli ulcer foto's.

Ik wil alle Nederlandse en Ghanese studenten en de studenten van andere nationaliteiten bedanken die op wat voor manier dan ook hebben geholpen met de studies en de patiëntenzorg. Daarbij wil ik expliciet noemen Rachael Bonawitz, en Meys Cohen voor je gezellige company, de studie die je hebt gedaan, en voor het helpen opvoeden van Emma, onze plassende waakpuppy. Jannes Sipkens dank ik voor de nare klus van het invoeren van alle audiometrie data, en Bram Zuur voor het plotseling enthousiast oppakken van de PKPD studie, en voor de ontzettend gezellige tijd die we hebben gehad.

Beste Gerrit en Maaike van Herwijnen. Dank voor het vervoeren van medische spullen naar Ghana, en voor jullie aandacht en vriendschap toen ik in Ghana was.

Beste Dr. Wim van Brakel and dear Dr. Ruth Butlin. Thank you for guiding my very first research project at The Leprosy Mission in India. You have laid the foundation for my further research activities.

Beste Dr. Kees Halma, mijn opleider in het MCL, Leeuwarden. Een perifeer ziekenhuis en onderzoek –wat niet in die kliniek is uitgevoerd– gaan soms moeilijk samen. Dank u voor de steun die ik toch altijd heb gevoeld. Beste Loek de Heide, mijn nieuwe opleider. Door de bezigheden met dit proefschrift heb ik steeds het idee gehad dat ik mij niet voor de volle 100% kon geven voor de opleiding, de kliniek. Gelukkig zal dat binnenkort anders zijn. Beste Prof. dr. ROB Gans. Ik wil u bedanken dat u mij de mogelijkheid heeft geboden om mijn opleidingsplek te bevriezen, zodat ik eerst dat wat ik graag wilde, het onderzoek, kon doen.
De collega's arts-assistenten in het Medisch Centrum Leeuwarden wil ik bedanken voor de collegialiteit en de mogelijkheden tot ruilen van diensten en praatjes in verband met muziekoptredens of deadlines. Bedankt ook internisten, intensivisten, longartsen, cardiologen, geriaters, medisch microbiologen, chirurgen, verpleegkundigen, secretaresses, assistentes en medisch fotografen voor de samenwerking.

Dear Martina Bührlen, a friend like you is hard to find. I can't imagine how it would have been if you wouldn't have been there, in Agogo. Thank you for sharing your house with me every week again, for the laughter and the tears, for the sports and the food.
Dear Katrin Fähndrich, after Martina went, you just let me, my friends and family, into your house without any hesitation. You are just the best!
Dear Virginie Dejoye, thank you for your friendship and for sharing your house and food with us during my last visit to Ghana.
Dear Cristophe Carillon, Alison Ely and Susanne Abels, thank you for your company. All of you shared your houses with me for a shorter or longer period of time. I am very grateful for that.
Dear Chris and Elize Schütte, thank you for your friendship and support during our gatherings, when I was in Kumasi.
Dear Marie-Anne Franqueville. I am honored that you allowed me to use some of your drawings to illustrate my thesis. Thank you so much!

Beste Auke-Willem en Feikje Kampen. Bedankt voor jullie vriendschap en gesprekken. Muziekvriend AW, het afscheidslied zal ik nooit vergeten. Of ik nou in Ghana of in Leeuwarden woon, we zien elkaar niet vaak – maar als we elkaar zien is het goed. Dat ik nu 10x bij je ben geweest om de omslag van het boekje aan te laten passen heeft daar trouwens verandering in gebracht. Misschien dat ik nu aan het lezen van jouw boek toekom! Feikje, bedankt voor het ons altijd maar weer voorzien van een van de eerste (?) levensbehoeften.

Beste paranimf, Sandra. Ik weet nog dat je mij in Emmen vroeg of ik het meende, dat ik iemand zocht om mee naar Brazilië te gaan. Jammer dat het ons niet gelukt is iets in Ghana te plannen, ook al hadden jullie al bijna geboekt. Ook al toon ik soms weinig commitment, de vriendschap zal voor mij altijd blijven.
Beste paranimf, Esther. Bedankt voor je vriendschap hier in Leeuwarden. Het was fijn om samen onderwijsdagen voor te bereiden en onszelf te motiveren tot werk, wetenschap en meer. Bedankt voor het mij uit bed bellen en voor de gezellige maaltijden.

Beste onderbuuf, Aafke Stapert. Bedankt dat je nooit klaagt, bijvoorbeeld over mijn getoeter. Bedankt voor je maaltijden en massages toen het schrijven bijna ten einde liep, maar ik ook bijna ten einde liep..

Christengemeente Buitenpost, bij jullie voel ik me thuis als ik er weer ben. Bedankt voor het meeleven vóór, en tijdens het project.

Naast de wetenschappelijke en klinische inspanningen heb ik gemerkt dat ik niet zonder muziek kan. Ik dank alle leden van Muziekvereniging Joost Wiersma, Harmonie'67 en de andere ad hoc muziekvrienden voor jullie aandeel hierin. Muziek is een van de mooiste dingen die bestaan.

Ferdinand van Nispen, als jij niet op mijn weg gekomen was dan was er geen lay-out geweest. Ik had het niet kunnen doen tussen mijn bedrijven door, en ik weet niet wie het anders zo snel nog voor elkaar zou hebben gekregen. Mijn hartelijke dank.

Lieve Pap en Mam. Bedankt voor de liefde en de steun die ik van jullie heb gekregen. Jullie hebben mij uiteindelijk de mogelijkheden gegeven om te doen wat ik wilde doen, mijn waardering hiervoor is groot. Het halen en brengen naar Schiphol, het pakken, verslepen en wegen van koffers en monsters, het regelen van grote en kleine

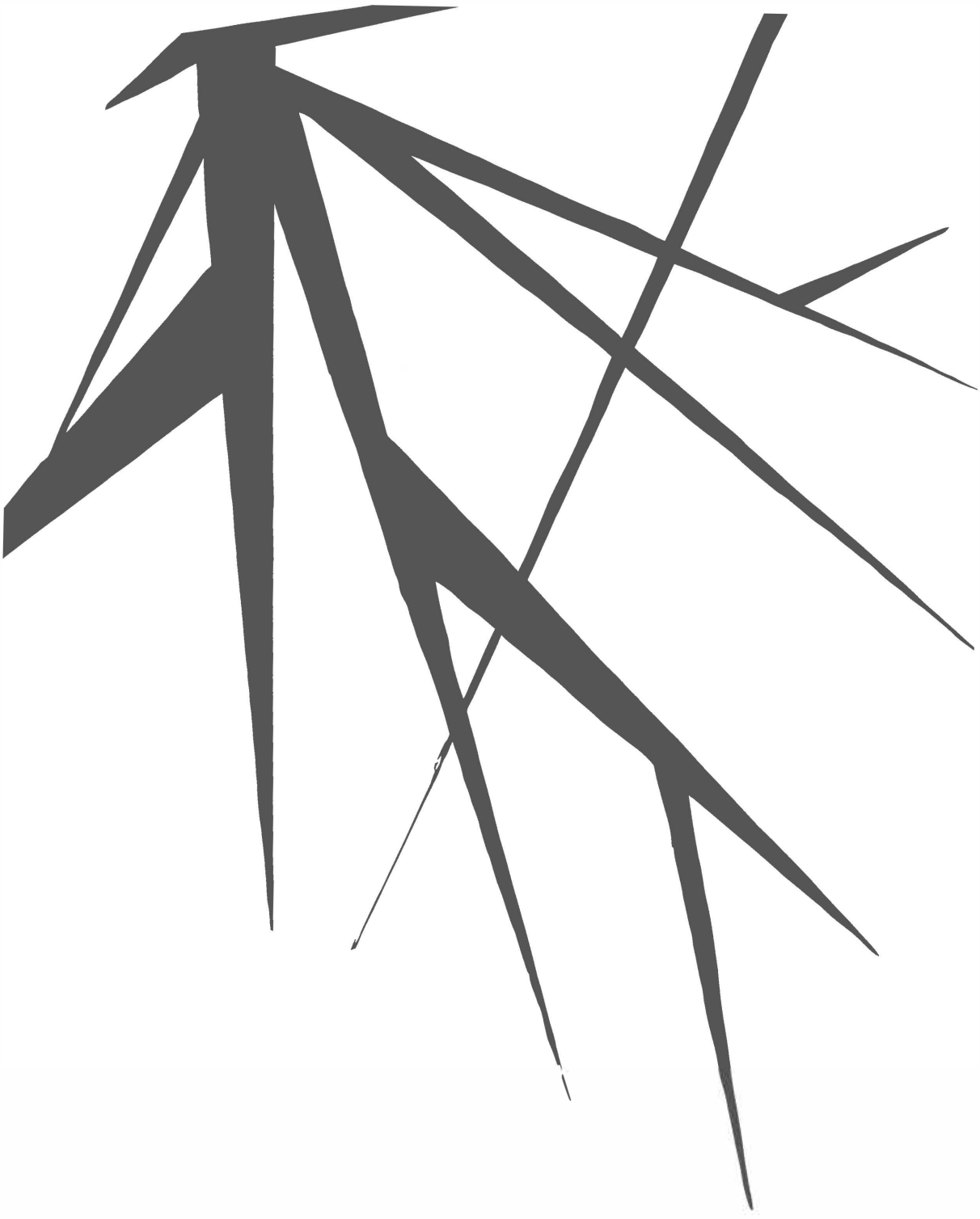
dingen, niets was teveel. Beste Pap, ik weet nog dat je mij vroeg of ik niet toch maar meteen aan de opleiding tot internist zou gaan beginnen toen die mogelijkheid er was. Ik weet dat pap het beste met mij voor heeft – gelukkig is er niets ernstigs gebeurd daar in het zwarte Afrika. Ik weet niet precies hoeveel dagen of weken het pap heeft gekost om alle oppervlakte sheets in te scannen, maar ik weet wel dat het er vele moeten zijn geweest. Gezien ons beider perfectionisme zal de betrouwbaarheid van de oppervlakteberekeningen waar we daarna samen weken aan gewerkt hebben, niet slecht zijn. Beste Mam, mam weet hoe het is om voor langere tijd in een andere wereld te leven en hoe moeilijk het is om weer terug te komen. Mam wist dat dit mijn diepste wens was, en heeft mij hier daarom altijd in gesteund, bedankt hiervoor. Ik heb het heel erg gewaardeerd dat jullie mij kwamen bezoeken, en ik zie ons nog in de bush fufu en vis met de handen eten. Pap heeft de vaccinaties overleefd en mam de buikloop. Wat was het fijn dat ik weer in Nederland was toen pap ziek werd. Ik hou van jullie.

Beste Thelly, bedankt voor de interesse en de liefde voor de ander die Thelly altijd met zich meedraagt. Het meedenken en de steun heb ik erg gewaardeerd. Bedankt voor het tijdelijke onderdak toen ik tussen mijn reizen door Nederland was. Het was ontzettend leuk om Thelly mee te hebben op mijn laatste reis naar Ghana. Ook mama Gré en oom Albert, jullie altijd warme belangstelling en zorgen heb ik op prijs gesteld.

Een heel aantal vrienden en familie heb ik niet genoemd. Toch zijn velen meelevend geweest, waarvoor dank. Dit heeft mij goed gedaan.

Lieve Janneke en Elske, wat is het fijn om een zus en een zusje te hebben. Bedankt voor jullie meeleven. Beste Ronald en Wouter, het is fijn om af en toe eens te horen wat iemand die buiten ons gezin is opgegroeid, er nou van vindt. In Canada heb ik heel wat tijd doorgebracht in de basement van Pompeblêd Poultry om te werken aan de database – Jan, bedankt. Els, het is leuk dat jij de onderzoekswereld kent, zo kunnen we daar af en toe eens samen over bomen en onze verbazingen uitspreken. In Ghana zeggen ze dat de kinderen van je zus je eigen kinderen zijn. Dat voelt inderdaad soms zo. Jiska, Daniël, Caleb, Gideon, Jesse & Lydia en Simon; ik hou van jullie allemaal.

Leeuwarden, februari 2012



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de Zeeuw J, Duggirala S, Nienhuis WA, Abass KM, Tuah W, Omansen TF, van der Werf TS, Stienstra Y. Serum levels of Neopterin during antimicrobial treatment in *Mycobacterium ulcerans* infection. *Submitted.*

Presentation list

Oral presentation: Reliability of the WHO 'disability' grading system. Congress 'International Workshop on Measuring Disablement in Relation to Leprosy', **2002** Nov, New Delhi (IN)

Poster: Reliability of the WHO 'disability' grading system. Studentencongres Groningen, **2003** June, Groningen (NL)

Oral presentation: Antimicrobial treatment for early, limited *Mycobacterium ulcerans* infection: a randomised controlled trial – preliminary findings. WHO annual meeting on Buruli ulcer, **2008**, March, Geneva (CH)

Poster: Pharmacokinetics of rimfampin and clarithromycin in patients treated for *Mycobacterium ulcerans* infection in Ghana. ICAAC/IDSA, **2008** Oct, Washington (US)

Oral presentation: Antimicrobial treatment for early *Mycobacterium ulcerans* infection. NBUP national meeting on Buruli ulcer, **2008**, Accra (Ghana)

Oral presentation: Antimicrobial treatment for early, limited *Mycobacterium ulcerans* infection: a randomised controlled trial and paradoxical responses during and after treatment. WHO annual meeting on Buruli ulcer, **2009**, March, Cotonou (Benin)

Best oral presentation: Antimicrobial treatment for early, limited *Mycobacterium ulcerans* infection: a randomised controlled trial. Wetenschapssymposium MCL, **2010** Febr, Leeuwarden (NL)

Oral presentation: Antimicrobial treatment for early, limited *Mycobacterium ulcerans* infection: a randomised controlled trial & paradoxical responses during and after treatment. Tropical Paediatrics Spring Congress, **2010** March, Zeist (NL)

Oral presentation: Premature atherotrombose en ptosis (MERFF syndrome), regional meeting on vascular medicine, **2010** Oct, Martini Ziekenhuis Groningen (NL)

About the author

Wilhelmina Anjelina Nienhuis (Willemien) was born on Thursday the 6th of April 1978 in Buitenpost, a town in the northeast of the province of Friesland, the Netherlands. She followed secondary education at 'het Lauwers College' in the same town, and started studying medicine in 1996 at the University of Groningen. After completing her internship in the Medical Center of Leeuwarden, in 2002 she conducted a doctoral research in The Leprosy Mission Hospital Purulia, West-Bengal, India, concerning *measuring impairment caused by leprosy: inter-tester reliability of the WHO disability grading system*, supervised by Dr Ruth Butlin and Dr Wim van Brakel. Here, she discovered that by performing clinical research she could combine her love for medicine and her wish to improve patient care on a larger scale. She presented the results of the study in New Delhi (India). She received her Master of Science and graduated as medical doctor in 2003. After 1½ years of residency in 'het Scheper Ziekenhuis Emmen' (Dr Frank van der Kleij), from 2005 to 2008 she coordinated a randomized controlled trial on *Drug treatment for Buruli ulcer disease* in Ghana, supervised by Prof Dr Tjip van der Werf and Dr Ymkje Stienstra. This study – with combined side studies – resulted in this thesis. She presented results of the studies in Cotonou (Benin) and at the WHO Buruli ulcer conference in Geneva (Switzerland). In 2009 she started her specialization internal medicine at the Medical Center of Leeuwarden supervised by Dr C Halma and later Dr L de Heide. From September 2012 she will continue the specialization at the University Medical Centre of Groningen under supervision of Prof Dr ROB Gans.